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Studies of Chloride Channels In Airway Epithelial Cells

by

Dan Liu

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

in

Experimental Medicine

Department of Medicine

Edmonton, Alberta

Fall, 1995

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FACULTY OF GRADUATE STUDIES AND RESEARCH

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Date: October, 1995

DEDICATION

to

MY PARENTS

Xin-yi Liu and Qin-lian Li

and

My supportive husband Nan Huang

My lovely son Fei Huang

Without whose love and understandanding

this thesis would not have been written

ABSTRACT

Epithelial apical membrane Cl^- channels were isolated and incorporated into giant liposomes used for single channel recording. Three Cl^- channels were most commonly observed; a linear 20 pS Cl^- channel, an outwardly rectifying Cl^- channel and a large conductance Cl^- channel. The biophysical characteristics of these three channels showed that they were similar to those observed in native epithelial tissues, indicating that the biochemical procedures for the isolation and reconstitution of ion channel proteins into giant liposome have not altered these ion channels.

It has been implied that many factors regulate Cl^- channels. Application of NaF and GTPγS activated a silent Cl^- channel from bovine tracheal epithelium with conductance of 100-120 pS. The channel was inhibited by the stilbene disulphonates SITS and DNDS. The activity of the channel was not affected by alkaline phosphatse, nor by okadaic acid and calyculin A, which are specific inhibitors of protein phosphatases type I, and A_2, therefore, the involvement of protein phosphatases in the regulation of the Cl^- channels was excluded in this preparation. The channel could be activated by GTPγS or by NaF in the presence of the phospholipase A_2 inhibitor, quinacrine, indicating that phospholipase A2 is not involved in regulation of this channel. The activation of the Cl^- channel by GTPγS didn't require the presence of ATP, indicating a direct activation of Cl^- channels by G-proteins.

The effects of pH on the activity of a large epithelial Cl^- channel from bovine tracheal epithelial cells were examined. Alkaline pH had no appreciable effect on channel

probability, and raised the voltage required to open the channel. This study suggested that at least one charged group on the channel protein, with pK_a of 6.09, may be responsible for its voltage dependence. Neutralization of these charges does not eliminate the voltage dependence, but changes the free energy differences between the closed and open states in the absence of an electric field. The lack of channel conductance changes in a wide pH range suggested the protonation site is far away from the channel permeation pathway.

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LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
AP	alkaline phosphatase
ATP	adenosine triphosphate
BCA	bio-rad protein concentration assay
cAMP	adenosine 3',5'-cyclic monophosphate
CF	cystic fibrosis
CFPAC-1	pancreatic carcinoma cell line from a patient with cystic fibrosis
CFTR	cystic fibrosis transmembrane regulator
CHAPS	3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate
CHO	chinese hamster ovary
DB	dialyzing buffer
dH_2O	deionized water
DIDS	4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid
DMSO	dimethylsulfoxide
DNase	desoxyribonuclease
DNDS	4,'4-dinitrostilbene-2,2'-sulfonic acid
EDTA	ethylenediaminetetraacetic acid
EGS	ethylene glycol solution

F	faraday constant
FBS	fetal bovine serum
f(V)	voltage-dependent open probabity
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HM	homogenization medium
H_2DIDS	4,4'-diisothiocyanatodihydrostilbene-2,2'-disulfonic acid
g	acceleration due to gravity
GHK	Goldman-Hodgkin-Katz
G-protein	guanine nucleotide binding protein
GTP	guanosine 5'-triphosphate
GTPγS	quanosine-5'-O-(3-thiotriphosphate)
GΩ	resistance; giga-ohms
ΔG	free energy difference between voltage-dependent and voltage-independent components
k	Boltzmann's constant ($1.38*10^{23}$ J/°K)
MEM	minimum essential medium
M199	medium 199
Na^+-K^+ ATPase	sodium-potassium-adenosinetriphophatase
N_h	the number of proton binding sites
N_z	the number of charges which traverse the membrane during the transition between the open and closed states

ORCC	outwardly rectifying chloride channel
P_m	a pH-independent component of open probability
pK_a	dissociation constant of acidic component
PKA	cAMP-dependent protein kinase
PKC	protein kinase C
P_{max}	maximum open probability
PMSF	phenylmethylsulfonyl fluoride
P_o	open probability
pS	conductance; picosiemens
PTX	pertussis toxin
R	gas constant
SDS	sodium dodeyl sulfate
SITS	4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid
T	absolute temperature in degree Kelvin
TEA	tetraethylammonium chloride
TRIS-HEPES	[Tris(hydroxymethyl)aminomethane]-N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
VDAC	voltage-dependent anion channel
V_m	membrane voltage
V	voltage; volts
V_o	the voltage at which half of channels open

X	acidic groups
V_R	Reversal potential
W	voltage-independent free energy component

CHAPTER 1 INTRODUCTION

1.1 Airway epithelial cells and mucociliary clearance

The airway epithelium is a pseudostratified columnar structure, which contains three main cell types: ciliated, goblet and basal cells. The ciliated and goblet cells extend from the basement membrane to mucosal surface. Approximately 250 cilia cover the apical surface of each ciliated cell. Ciliated cells are the ion-transport cells (Welsh 1987). Normally, ciliated cells are more numerous than goblet cells. The basal cells are near the basement membrane and appear to be germinal cells that develop into ciliated or goblet cells via intermediate cells.

The concept that the epithelium is covered by a layer of fluid consisting of a superficial mucous layer and a periciliary fluid layer began early (Lucas and Douglas 1934). The submucosal glands produce most of the mucus (Basbaum 1986) The mucous layer may cover the airways like a continuous blanket or may float like islands over the cilia (Hulbert et al. 1982). In any case, the mucus traps and carries inhaled particulates. How well the mucus performs this task depends on its quantity and its viscoelastic properties, since the particles must adhere to the mucus, and the mucus must interact mechanically with the tips of the underlying cilia. The periciliary fluid is less viscous and bathes the cilia; the depth of this layer approximates the length of the cilia. Periciliary fluid provides a watery environment in which the cilia beat freely, with only their tips projecting to contact the mucus layer. The depth of periciliary fluid seems critical to mucociliary

clearance: a deep layer will uncouple cilia beating with movement of the mucous layer, and too shallow, a layer will prevent the optimal pattern of ciliary beating.

Mucociliary clearance is the mechanism by which mucus and inhaled debris are removed from the airway. Figure 1.1 shows some of the important features required for effective mucocilliary clearance.

1.2 Ion transport and apical membrane chloride channels in airway epithelial cells

Airway surface liquid is critical for normal function of mucociliary clearance, and active ion transport is thought to provide the principal driving force for liquid movement across the airway epithelium. Surface liquid volume depends upon the magnitude of imbalance between active ion secretion and absorption across this structure. Figure 1.2 hows a model that describes the major components that control electrochemical behavior in airway epithelium, including the current understanding of how airway epithelial cells secrete chloride and absorb sodium. In summary:

1) Chloride ions enter the cell across the basolateral membrane via an electrically neutral co-transport process, coupled to Na^+ and possibly K^+.

2) Chloride ions exit passively through apical membrane Cl^- channels, moving down a favorable electrochemical gradient. The factors that regulate the apical membrane permeability to Cl^- control the rate of transepithelial secretion.

3) Sodium ions enter the cell passively at the apical membrane through Na^+ channels, and at the basolateral membrane coupled to Cl^-.

4) Sodium ions exit across the basolateral membrane via the Na^+-K^+ ATPase. This enzyme provides the energy for transepithelial Cl^- secretion by maintaining a low intracellular Na^+ concentration.

5) Potassium ions exit passively through a basolateral membrane K^+ channel. The basolateral K^+ conductance and K^+ gradient hyperpolarize the cell, providing the electrical driving force for Cl^- to exit.

The major driving force for ion movements is Na^+-K^+ ATPase in the basolateral membrane, which produces low intracellular Na^+ concentration and a high intracellular K^+ concentration. The intracellular chloride ion concentration is elevated by a passive Na^+-K^+-$2Cl^-$ cotransport system, driven by the high interstitial sodium. This high intracellular chloride concentration, together with the electrical gradient, favors passive chloride exit through the apical membrane via chloride conductance.

It is clear that the epithelium can actively secrete Cl^-, which then drives fluid secretion. The apical membrane Cl^- conductance provides major control of transepithelial Cl^- movements and the apical membrane chloride channel is therefore a key site for the control of salt and fluid secretion across many epithelia (Frizzelle and Halm 1990; Shoemaker et al. 1986, Welsh 1986a).

1.3 Classification of apical membrane chloride channels in airway epithelium

There are several types of Cl^- channels with different characteristics. During the last decade, the single channel recording technique has been used to identify some of these channels. According to permeability sequences, at least 4 groups of apical membrane chloride channels have been identified in mammalian airway epithelium.

1.3.1 Small chloride channels

The small chloride channels have a mean linear conductance of ≈ 10 pS, and are not voltage-sensitive (Vaughan and French 1989). Other channels have been reported in canine tracheal epithelium (Shoemaker et al. 1986), and they are 10 pS, linear and voltage sensitive channels. since their open probability increases with depolarization. The small chloride channels of epithelia deserve much more attention because despite their low conductance they are present in a large number and can account for a significant fraction of the total cell membrane conductance. In human nasal epithelium, based on a survey of more than 170 different channels from 26 normal subjects, Duszyk et al 1989 found that small channels comprised 15% of the total chloride channels observed in human nasal epithelial cells, but suggested that this number was probably, significantly underestimated. The size of the channels make them difficult to study, not only because of the noise level of patch-clamp recordings, but also because they are often obscured by other channels. The halide permeability sequences of the small Cl^- channel obtained in human airway epithelial cells was $Cl^- > I^- > Br^- > F^-$ for the 10 pS channel (Wilk-Blaszczak et al 1992). The

cystic fibrosis transmembrane conductance regulator (CFTR) is a linear, voltage-insensitive small conductance Cl^- channel which is within the range of conductances of the 10 pS channels (Riordam et al 1993). However, expression of normal CFTR in several different cell lines produced whole cell current with the permeability sequence $Br^- > Cl^- > I^- > F^-$ (Anderson et al 1991), and the relative permeability ratios were much larger than the one observed by Wilk-Blaszczak (1992). The exact contributions of these voltage-insensitive 10 pS channels to the currents in normal and cystic fibrosis airway epithelial cells remain uncertain.

1.3.2 20 pS chloride channels

The 20 pS non-rectifying channel has been identified in airway epithelia (Frizzell et al., 1986; Shoemaker et al.,1986; Duszyk et al.,1990). The reports by Shoemaker et al and Krouse et al. both suggest that the channel is voltage-sensitive, opening more with depolarization. However, the study by Duszyk et al.,(1990) included a complete kinetic analysis over a wide voltage range and found no voltage or calcium sensitivity. They found the 20pS channels to be the most prevalent ion channels in nasal epithelia, accounting for a large fraction of the total chloride conductance. They have also shown the existence of a sub-group of non-rectifying channels, which have a mean conductance of $\approx$ 30 pS. The study of anomalous mole fraction behavior showed that the conductance through the 20 pS channel was reduced below the linear prediction with an asymmetric mixture of ions. Therefore, the 20 pS Cl^- channel probably contains a multi-ion pore (Wilk-Blaszczak and French 1992). It has a weak binding site inside the channel (about 3 kT), and its kinetic

behavior could be described by a model having three open and three closed states (Duszyk et al., 1990).

1.3.3 Outwardly rectifying chloride channels

The outwardly rectifying chloride channels (ORCC) are most widely studied among epithelial ion channels. Their most common feature, other than rectification, is their activation by second message chemicals, particularly cyclic AMP. By definition, they have voltage-dependent conductances, which increase at more positive membrane potentials to give outward rectification. This raises a physiological question about its relevance, since an outwardly rectifying chloride channels in apical membrane of an epithelium will pass current most effectively in the opposite direction to the normal chloride flux. A possible explanation of this behavior is that the depolarization of membrane potential will decrease the electrochemical gradient driving chloride out through the apical membrane, so that outward rectification might counteract this effect, by providing a negative feedback regulation at the single channel level.

In airway epithelia, rectifying chloride channels have been reported in human tracheal (Frizzle et al. 1986; Welsh 1986a), human nasal (Kunzelmann et al 1989, Duszyk et al 1989) and canine tracheal epithelia (Shoemaker et al 1986; Welsh 1986b, 1987). There is also a report of rectifying chloride channels from bovine tracheal epithelium reconstituted into planar lipid bilayers (Valdivia et al 1988). Conductances in dissociated epithelia range from about 30 pS (Welsh, 1986b, 1987) to about 50 pS (Kunzelman et al. 1989) and 70 pS in reconstituted channels. The regulation of open probability for this

type of channel has been studied intensively because of its relationship to cystic fibrosis (Li et al. 1989). ORCC are regulated by CFTR. ORCC is defective in cystic fibrosis, but with normal CFTR, ORCC functions normally. The channel appears to be regulated by both protein kinase A- and protein kinase C-dependent mechanisms, with intracellular calcium concentration affecting the balance between the two processes (Vaughan and French 1989).

1.3.4 Large chloride channels

The large conductance channels are clearly the most voltage-sensitive among all epithelial chloride channels. They have been reported in a variety of tissues. such as canine tracheal (Shoemaker et al., 1986), mouse alveolar (Krouse et al 1986) and human nasal epithelium (Duszyk et al., 1989a). The estimated conductances of the large channels varied from 250-450 pS, and, most were in the range of 350-400 pS. A problem with the estimation of the conductance and linearity of these channels is the occurrence of sub-conductances. It is not clear whether the appearance of sub-conductances in the channels reflects groups of smaller channels acting cooperatively, multi-barrelled channels, or single channels with variable pore properties. However, the repeated finding of a conductance of ≈350 pS in a wide range of tissues suggests a single type of channel, rather than groups of cooperative channels (Vaughan and French 1989, for review). The voltage sensitivity of the open probability of the large channels is also well established with most investigators reporting activation of the channels within the voltage of -10mV to + 10 mV. However, the voltage dependence of activation and inactivation is not well understood (Vaughan and French 1989). The most probable explanation of these results is that the channels are

inactivated at significantly negative membrane potentials, including the resting membrane potential of most epithelia, activated with depolarization to around zero voltage or above, and then inactivated with a time constant of up to several seconds. The large conductance Cl^- channels in native epithelia rarely open, making a detailed analysis of their properties difficult. For example, in human airway epithelial cells (Duszyk et al.,1989), this channel was found in 8 patches out of 495 studied (1.6%).

1.4 Regulation of chloride channels

1.4.1 Regulation of chloride channels by voltages

Shoemaker et al (1986) reported a voltage-dependent Cl^- channel in canine tracheal epithelial cells. This channel was activated at voltages more negative than +60mV, and inactivated at potentials of +80mV to 120mV. Following inactivation at more positive potentials, channel activity resumed when the membrane potential was returned to the hyperpolarized potential of -100mV. In contrast, Schoumacher and coworkers(1987) have shown that Cl^- channels from cultured human tracheal epithelial cells can be activated by voltage pulses to depolarizing voltages of $\geq$+40mV. Depolarizing voltages applied to cell-free membrane patches activate chloride channels. Activation is proportional to the duration and magnitude of the depolarizing voltage (Welsh 1989). In cell-attached patches, membrane depolarization does not activate chloride channels, but following excision, depolarization of equivalent magnitude activates

the channels. Activation in cell-free patches by depolarization is readily reversed when hyperpolarizing voltages are applied to the patch; channels may be repeatedly switched back and forth between activated and inactivated states by changes in membrane voltage. This reversibility suggests that activation does not result from the removal of an inhibitory factors from the channel environment. Several observations indicated that depolarization-induced activation was not a physiologic process. First, it was not observed in cell-attached patches. Second, Cl^- secretagogues produced only relatively small changes in apical membrane voltage (Welsh 1987). Third, in native airway epithelia changes in apical voltage caused by imposition of transepithelial currents did not affect apical conductance(Welsh 1985).

1.4.2 Regulation of chloride channels by cAMP, PKA and PKC

Many agonists such as β-adrenergic agonists, prostaglandin E_2 (Smith 1990) stimulate an increase in intracellular cAMP in airway epithelial cells along with an increase in chloride secretion: In addition, membrane-permeable cAMP analogues mimic this secretory effect (Welsh 1986b; Frizzell 1986). These studies indicate that cAMP acts as an intracellular messenger for receptor-mediated chloride secretion.

The role of cAMP in channel regulation has been investigated more directly in cultured airway cells using the patch-clamp technique. In cell-free patches, cAMP-dependent protein kinase(PKA) activates Cl^- channels in the presence of cAMP and ATP (Li et al 1988; Schoumacher et al 1987). The mechanism of action is depicted in Fig. 1.3. PKA exists predominantly in inactive form with two regulatory and two catalytic subunits

combined. Binding of four cAMP molecules to the regulatory subunits causes the release of the catalytic subunits which have phosphorylation activity. In airway cells, the catalytic subunits presumably phosphorylate the channel itself, or a membrane-associated protein coupled to channel activity. The mechanism of activation suggests that the channel, or regulatory protein, exists in phosphorylated and dephophorylated states corresponding to activated and inactivated states of the channel, respectively. The probability of chloride channels being open may depend on a balance of competing kinase and phosphate activities (Levitan 1985; Brautigan 1988; Kume et al 1989).

Other kinases may also regulate the activation of Cl^- channels (Fig.3). Li and co-workers (1989) investigated the effects of protein kinase C (PKC) on channel activation. Cell-free membrane patches excised from cultured canine and human airway cells were exposed to PKC in the presence of ATP and diacylglycerol. When $[Ca^{2+}]$ was <10 nmol L^{-1}, PKC activated Cl^- channels in the majority of cell-free patches. In control patches(when PKC was not present), no Cl^- channels were activated in any of the patches. The effect of PKC in the presence of higher Ca^{2+} concentration differed. With $[Ca^{2+}] \geq 1$ µmol L^{-1}, PKC inactivated Cl^- channels. Furthermore, after exposure to PKC at high $[Ca^{2+}]$, the channels could not be activated by depolarization. In control patches exposed to high $[Ca^{2+}]$ (without PKC), the channels were activated by depolarization. These findings indicate that PKC may have both activating and inactivating effects on Cl^- channels in airway cells depending on the local Ca^{2+} concentration. Channel activation by PKC required low $[Ca^{2+}]$; inactivation by PKC requires a higher $[Ca^{2+}]$. Activation by PKC appears to have a shorter response time than inactivation.

1.4.3 Regulation of chloride channels by intracellular calcium

Although it is known that an increased intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$)stimulates airway Cl^- secretion (Frizzell et al 1986), the cellular mechanism and the channels involved have not yet been identified. Frizzell and colleagues (1986) demonstrated Cl^- channel activation by the calcium ionophore A23187 in cell-attached patches of airway epithelial cells and suggested that the mechanism of action is not through direct activation of the channels by Ca^{2+} for the following reasons: 1) channel activation on excised patches is not consistently observed over a wide range of Ca^{2+} (Welsh and Liedtke 1986c; and Li et al. 1989). 2) the onset of channel activation in excised patches exposed to Ca^{2+} may be delayed for several minutes (Frizzell 1987). Thus it is still unclear how increases in intracellular $[Ca^{2+}]$ lead to Cl^- secretion. Intracellular $[Ca^{2+}]$ may stimulate phospholipase A_2 activity which releases arachidonate and may lead to prostaglandin-mediated Cl^- channel activation. Intracellular $[Ca^{2+}]$ may stimulate PKC, activity and thereby, activate Cl^- secretion (Li et al. 1989; Hwang et al. 1989). Increases in intracellular $[Ca^{2+}]$ may also enhance Cl^- secretion by activation of basolateral K^+ channels (Smith et al. 1990; McCann and Welsh 1990). This would increase the electrical driving force for Cl^- efflux via already activated Cl^- channel.

1.4.4 Regulation of chloride channels by swelling

Chloride currents associated with cell swelling have been described in a variety of epithelial cells. In whole-cell patch clamp recordings, they can be induced by an osmotic gradient that favors water entry into the cell. It has been described that increased membrane chloride conductance has been associated with such volume-sensitive currents

in human airway epithelium. Clear separation of volume effects from other intracellular signals has not always been possible. Cross-sensitivity to volume and voltage has been reported in several cases, with depolarization causing inactivation, although other volume-sensitive currents are not inactivated by voltage. The swelling-induced Cl^- current in epithelia has been most commonly associated with an outwardly rectifying chloride channel of about 50 pS conductance, based on the outwardly rectifying nature of the current, observations of discrete current jumps in whole cell records, and single channel records from swollen cells.

1.5 Methods for studying ion channels

1.5.1 Patch clamp technique

The development of patch-clamp techniques for studying ionic channels has virtually revolutionized the ability of physiologists to study the basic mechanisms of ionic transport. Basically, the method involves the use of glass pipettes with fire polished tips (.5-1.0μm diameter) filled with saline and connected to a current-voltage amplifier. The pipettes are pressed against the cell membrane and by applying a little suction, a high-resistance seal (about 10^9 ~10^{10} Ω) is formed which electrically isolates a small patch of membrane. In this manner, individual channel currents can be resolved.

An experimental advantage of patch clamp techniques is that four different recording configurations are possible, depending on the quenstion of interest, (see Figure

1.4 Hamil 1981). Single channel recordings can be obtained by using any of these three modes; cell-attached mold and excised modes (inside-out mode and outside-out mode). Excised patches are particularly advantageous for altering solutions, determining channel ionic selectivity, and distinguishing between regulatory mechanisms that are inherent in the plasma membrane and those which originate intracellularly.

1.5.2 Reconstitution method

Reconstitution method is a powerful tool for studying the properties of single ion channels. In this method, ion channel proteins are isolated and/or purified in biochemical procedures and incorporated into planar lipid bilayer membranes or giant liposomes suitable for single channel recordings, see figure 1.5. The goals of the method are twofold: 1) to determine channel behavior under conditions in which the bathing solution composition and lipid composition are highly controlled; and 2) to verify that the purified channels function normally.

It is clear that patch-clamp technique combined with reconstitution method provides investigators the ability to determine the properties of the ion channels, the structures which mediate the ionic conductances as well as to determine the factors which affect these channel proteins.

1.6 OBJECTIVES

The objectives of my research were to characterize the apical membrane chloride channels in airway epithelial cells. In particular:

1) To examine the biophysical characteristics of chloride channels isolated from epithelial cells, incorporated into giant liposomes; and

2) To investigate the regulation of a chloride channel from bovine tracheal epithelium by G-protein.

3) To examined the behavior of a large conductance chloride channel during asymmetric pH changes and identify titratable groups which affect the activity of the channels.

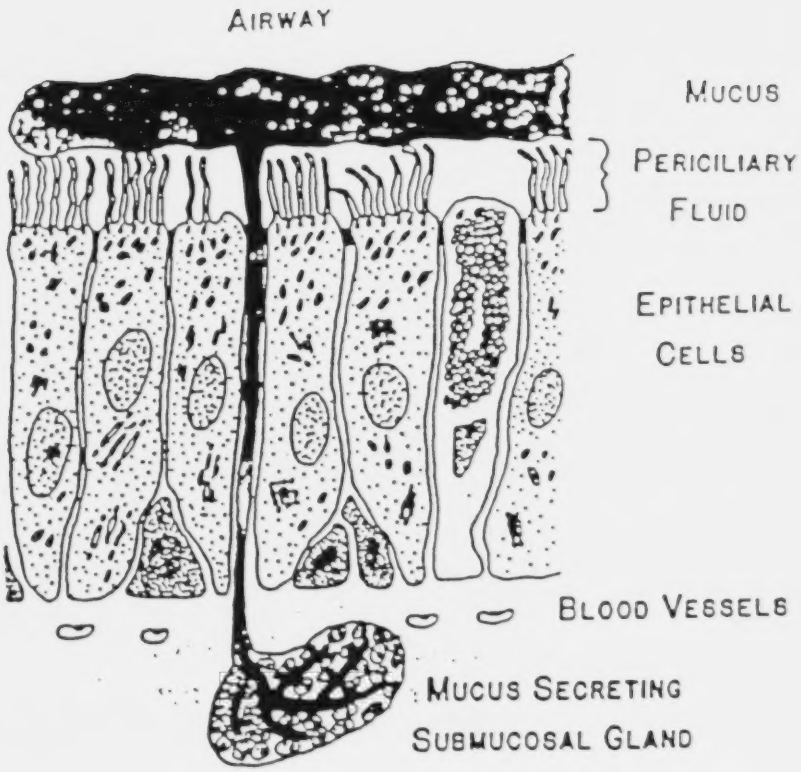

Fig.1.1 Schematic model of elements of mucociliary clearance apparatus in airway epithelium (adapted from Welsh 1987)

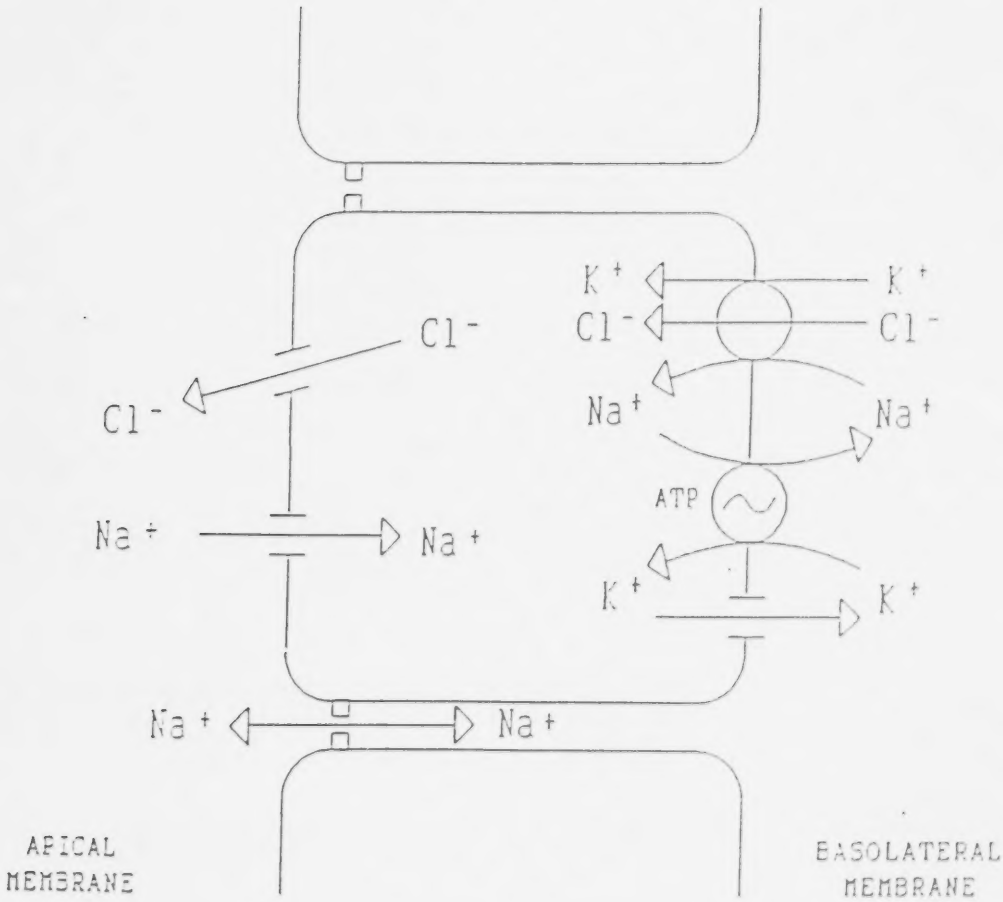

Fig. 1.2 A general model of ion transport across the airway epithelium (adapted from Welsh 1987)

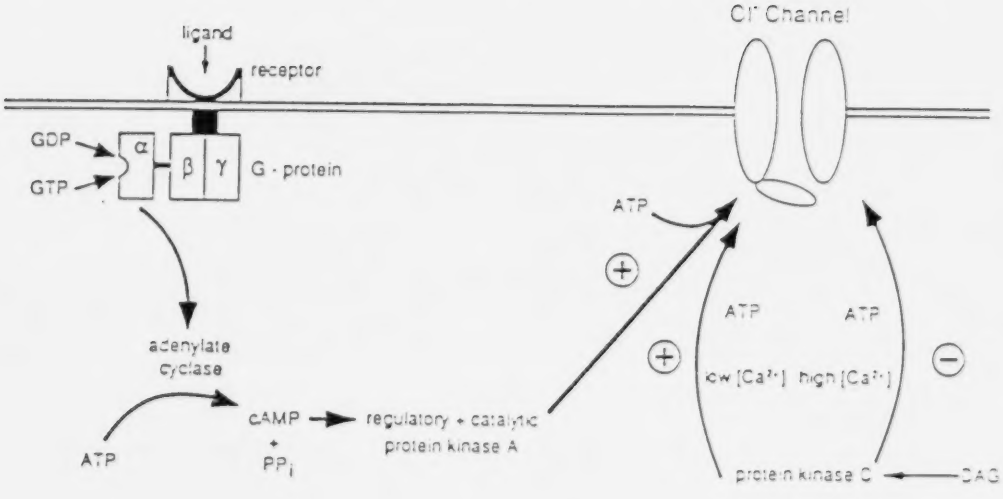

Fig.1.3 Model of cAMP-mediated Cl^- channel activation and the effect of PKC on channel activation (adapted from Smith, 1990)

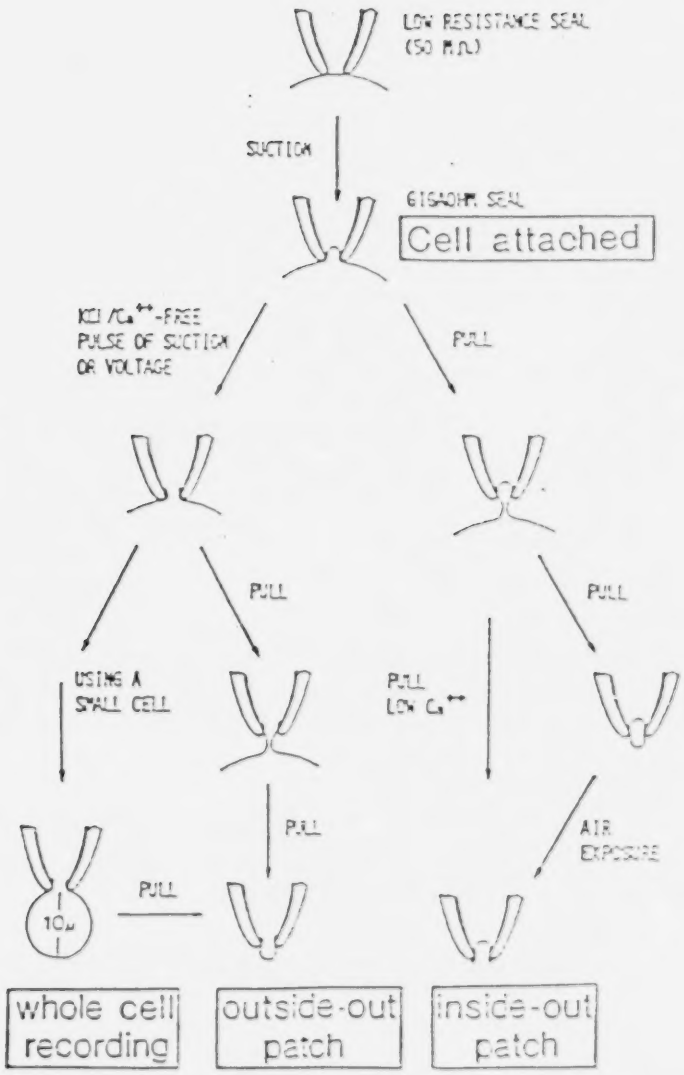

Fig. 1.4 Schematic presentation of the patch clamp technique (adapted from Hamil. 1981)

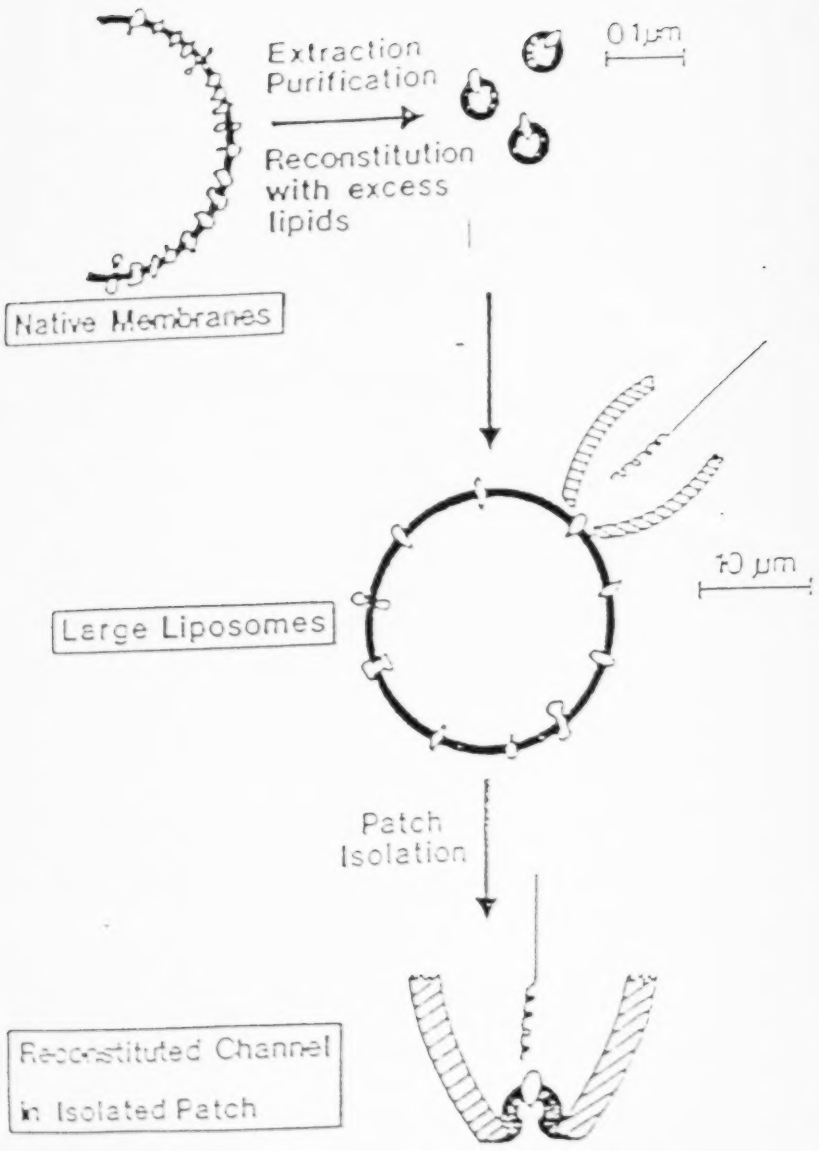

Fig. 1.5 Schematic diagram of the strategy for combining the patch recording technique with the general method of membrane protein isolation and reconstitution into liposome by detergent dialysis (adapted from Sakmann 1983)

CHAPTER 2. MATERIALS AND METHODS

2.1 Cell preparations

2.1.1 CFPAC-1

A pancreatic carcinoma cell line from a patient with cystic fibrosis was obtained from the American Type Culture Collection. Cells were plated on plastic tissue culture flasks (150cm^2 growing area) at 1×10^4 cell/cm^2, in M199 medium (Sigma), supplemented with 10% FBS and antibiotics (gentamycin 50μg/ml, streptomycin 100μg/ml, and penicillin-G 60μg/ml). Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in air, and the medium was changed every other day. After reaching confluence(~10 days), cells were used for the membrane preparation.

2.1.2 Bovine tracheal epithelial cells

Bovine tracheae were obtained from a local slaughterhouse. The tracheae were removed 10-15 min after death and immediately placed in ice-cold oxygenated Ringer's solution for transport to the laboratory. The tissues were washed in the Ringer's solution. Epithelial cells were obtained with enzyme digestion. Strips of mucosa and underlying connective tissues were removed, and treated with 0.1% protease in calcium-free minimum essential medium (MEM) at 4°C for 16-20 h. 0.01% deoxyribonuclease (Dnase) was added to the protease solution at 37°C for 1.5-2.0 h. The enzymes were neutralized with 10% fetal bovine serum (FBS), and cells were detached from the epithelial strips by

gentle mechanical agitation. After being washed in MEM with 10% FBS, cells were plated on collagen-coated Falcon Primaria plates, the isolated cells were used for patch clamp single channel recording at room temperature, usually within 0-3 day after plating.

2.2 Reconstitution of apical membrane chloride channel proteins

2.2.1 Preparation of apical membrane proteins

Apical membrane proteins were prepared from CFPAC-1 cell line and bovine tracheal epithelial cells. In some experiments, the confluent CFPAC-1 cells on plastic tissue culture flasks (150cm^2 growing area) were washed with ice-cold washing buffer consisting of 140mM NaCl, 0.1mM CaCl and 5mM TRIS-HEPES pH=7.4, and then scraped off the flasks with a cell scraper (Costar) into homogenization medium (HM) containing 60 mM manital, 0.1 mM CaCl and 5 mM TRIS-HEPES pH=7.4 with protease inhibitors (25μg/ml aprotinin, 10μg/ml Leupeptin, 10μg/ml pepstain and 175 μg/ml PMSF); In other experiments, eight bovine tracheas were used to prepare cell membrane proteins (26 preparations). The mucosa was scraped in the HM buffer. The scrapings were homogenized on ice and the homogenants were sequentially centrifuged at 3,000g for 10 min to remove cellular debris and nuclei, at 10,000g for 10 min to remove mitochondria, and 37,000g for 40 min to collect plasma membranes. In order to separate apical membrane from basolateral membrane, 10 mM of $MgCl_2$ was added to the suspension of plasma membrane and stirred gently for 1h at 4°C. Aggregated membranes (basolateral)

were then spun down(6,500g for 12 min) and the membranes remaining in the supernatant were pelleted with a high speed centrifugation (100,000g, 60 min). The pellets were resuspended in a buffer consisting of 100 mM NaCl, 10 mM HEPES and protease inhibitors pH=7.4 to produce a crude apical membrane preparation (fraction A1). Protein and enzyme assays were determined using a BCA assay (Bollag and Edelstein 1991), and protein concentration was adjusted to 1 mg/ml. Apical membrane concentration was enriched up to 34 times compared to the homogenate, as assessed by measuring the activity of alkaline phosphates (AP) in fraction A1. Similarly, there was a 3 fold enrichment of basolateral membrane in fraction B1, assessed by measuring the activity of Na/K-ATPase. Apical membrane proteins were subsequently solubilized with 8 mM CHAPS, and shaken at 4°C for 60 min. This material was then spun for 60 min at 43,000g. The supernatant containing the detergent-solubilized apical membrane proteins (fraction A2) was stored at 4°C.

In some experiments, fraction A2 was titrated to pH=10.8 with 0.1 M NaOH and incubate on ice for 30 min to remove membrane-bound proteins from the apical membrane preparation (Neubig et al 1979). Following neutralization with HEPES, the solution was spun at 150,000g for 40 min and the pellet was resuspended to give a partially purified apical membrane fraction. All steps in protein preparation were carried out at 4°C.

2.2.2 Preparation of giant liposome

Giant liposomes were prepared by a modification of the method described by Keller et al.(1988) and Riquelme et al (1990). Phosphatidylcholine from soybeam (type 2-

S, Sigma) and CHAPS was dissolved in deionized water, then mixed with the fraction A2 of proteins in the ratio of 2:5:3. The mixture was incubated for 1h on ice, following dialysis against 1000-2000 volumes of dialysing buffer (DB) consisting of 100 mM NaCl and 10 mM HEPES pH=7.4 for 24h using Spectrapor 3 dialysis tubing (Spectral). After dialysis, the mixture was ultracentrifuged for 60 min at 100,000g. The pellet was suspended in 75µl of ethylene glycol solution (EGS) containing 50 mM NaCl, 5 mM HEPES,and 5%(v/v) ethylene glycol. The suspension was then deposited as small drops (12µl) on a culture dish, and partially dehydrated for 4-5h at 4°C in a desiccator containing anhydrous $CaCl_2$. The samples were rehydrated by adding 14µl of 50mM NaCl on top of each dehydrated drop, at 4°C overnight in Petri dishes (containing wet towel paper on the bottom). The resulting giant liposomes were pipetted off the rehydrated drops, spun at 300g for 8 min, resuspended and deposited on Petri dishes for patch clamp single channel recordings.

2.3 Single channel recordings of patch clamp

Single channels recordings were obtained using the patch clamp technique as described by Hamill et al (1981). In this study, I used inside-out mode, which allowed me to control the solutions on both sides of ion channel protein. The extracellular side of ion channel protein was in contact with the pipette solution, and the intracellular side with the bath solution. Thus, I was able to change intracellular solution. Patch pipettes were made

from borosilicate microfilament glass using a two stage puller. The pipette tips were coated with Sylgard (Dow Corning) to reduce tip capacitance, and fire-polished. Pipette resistance was about 12 MΩ. Current was detected using a list EPC-7 patch clamp amplifier, and recorded on video tape. Pipette offset potentials were measured and corrected before forming a seal. Changes in junction potential at the reference electrode due to changing halide solutions in the bath were calculated from the Henderson equation (McInnes 1939). The potential displayed by the patch-clamp amplifier was corrected for the liquid junction potential to obtain the potential across the patch (Neher 1992). Exchange solutions were provided by a multiple pathway flow system with very small dead space to minimize exchange times. In some experiments, a piece of Teflon tube was slid onto the tip of a pipette, which allowed to transfer the pipette from one solution to another, without loosing a gigaohm seal. All potentials are reported relative to zero in the extracellular(pipette)solution, and positive currents are outwards throughout.

2.4 Solution and drugs

Standard pipette solution contained (mM) 140 CholineCl or NaCl, 1 $MgCl_2$, 1 $CaCl_2$ and 10 HEPES, pH=7.4. Standard bath solution contained 140 NaCl, 1 $MgCl_2$, 1 $CaCl_2$, and 10 HEPES, pH=7.4. In experiments with ion permeation, 140 mM NaCl was replaced by 140 mM of NaBr, NaI, or NaF. Variable mole-fraction behavior was studied using different proportions of Cl^- and F^- ions in a total 140 mM halide. For fluoride

activation studies, 70 mM of NaCl was replaced by 70 mM of NaF in the Bath. Calcium-free solution contained (mM): 70 NaCl, 70 NaF, 1 $MgCl_2$, 5 EGTA, 10 HEPES, pH=7.4.

Alkaline phosphatase (AP) from calf intestinal mucosa (Sigma) was dissolved to final concentration of 1000 U/ml, and dialyzed against 1000 volumes of saline for 24 h. Okadaic acid (Molecular Probe, USA) was dissolved in DMSO as 100 μM stock solution. Calyculine A (Sigma) was dissolved in 100% ethanol as 100 μM stock solution. Okadaic acid and Calyculin A (Sigma) was diluted with bath solution before use. Guanosine 5'-[γ-thio]triphosphate (GTPγs) (Sigma, St. Louis, Mo) was prepared at a concentration of 1 mM. 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid (SITS), and 4,4'-dinitrostilbene-2,2'-sulfonic acid, (DNDS) (Molecular Probes, USA) were dissolved in water at a concentration of 100 μM. Sodium vanadate was prepared as a 10 mM stock solution and used at a concentration of 1 mM. Okadaic acid, calyculin A, GTPγs, SITS and DNDS were used on the same day that they were prepared.

2.5 Data analysis

The procedures used for computer data analysis were based largely on those described by Colquhoun and Sigworth (1985). The half-amplitude criterion was used as a threshold to distinguish between open and closed states. Even durations were corrected for filter rise-time by a polynomial approximation. Distributions of open and closed times were created and the channel open probability was calculated. Only openings longer than

the filter dead-time were used to compute the mean channel current amplitude. Channel conductance and ionic selectivity was determined by fitting the current-voltage relationships with the Goldman-Hodgkin-Katz current equation (1) (Hille 1992).

$$I_{Cl^-} = P_{Cl^-} * \frac{EF^2}{RT} \frac{[Cl^-]_o * \exp^{EF/RT} - [Cl^-]_i}{\exp^{EF/RT} - 1} \qquad (1)$$

Where P_{Cl^-} is the permeability of Cl^-, the subscripts "O" and "i" denote the outside and inside solutions, F, R, and T are Faraday constant, gas constant and absolute temperature respectively. Reversal potentials were determined from current-voltage relationships for different anions, and the relative ion permeabilities were calculated from the following equation (2):

$$V_R = \frac{RT}{F} * In \frac{[Cl^-]_o + (P_A / P_{Cl})[A^-]_o}{[Cl^-]_i + (P_A / P_{Cl})[A^-]_i} \qquad (2)$$

Where P_A is the peameability of an anion A^-. The Permeability ratio P_{Na}/P_{Cl} was determined from the GHK current equation (3).

$$I = I_{Na^+} + I_{Cl^-} = g * E \frac{P_X[Na^+]_i * \exp^{EF/RT} - [Na^+]_o}{[Cl^-]_i * \exp^{EF/RT} - 1} + \frac{[Cl^-]_o * \exp^{EF/RT} - [Cl^-]_i}{[Cl^-]_i * \exp^{EF/RT} - 1} \qquad (3)$$

Where $g = \frac{F^2}{RT} * p_{Cl^-} * [Cl^-]_i$, $P_X = P_{Na}/P_{Cl}$.

*CHAPTER 3. STUDY OF HALIDE PERMEATION THROUGH THREE TYPES OF EPITHELIAL ANION CHANNELS AFTER RECONSTITUTION INTO GIANT LIPOSOMES**

3.1 Introduction

Movement of an ion through a channel involves the replacement of ion-medium interactions with ion-channel interaction. As ions permeate a channel pore, they "sense" the electrostatic forces generated by amino acids that line the pore. These interactions determine the selectivity of the ion channel (Hille 1992), and are often used as a criterion to distinguish between different groups of ion channels. Anion channels are usually characterized by determining their relative permeability to different halides (Halm and Frizzell 1992; Wilk-Blaszczak et al 1992). Another important feature of an ion channel is the number of ions which can be accommodated in the pore while they enter, cross, and leave the channel. Some insight into this phenomenon may be obtained by studies of mole-fraction dependence from concentration-dependent permeability ratios In a single-ion channel, conductance or reversal potential should vary linearly with the ratio of mixed ionic concentrations. In a multi-ion channel, conductance often reaches a maximum or minimum as a function of ionic ratio.

The main objective of this thesis research is to characterize the properties of the chloride channels of airway epithelium; in this study, I did a biophysical characterization of

* Part c of this chapter has appeared in *European Biophysics Journal* 22; 5-11. 1993

epithelia apical membrane Cl^- channels which had been isolated and incorporated into giant liposomes. Using the patch clamp technique, I demonstrated that the biochemical procedure for isolation of ion channel proteins has not altered ion channel activity. At least four groups of anion channels reconstituted into giant liposomes were found, and most commonly observed anion channels were previously demonstrated in the native tissues.

3.2 Experimental design

In this study, apical membrane chloride channel proteins were isolated from CFPAC-1 cell line. The isolated apical membrane channel proteins were incorporated into giant liposome system, and single channel recordings (inside-out mode) were used to characterize the anion channels. Halide permeabilities were examined by exchanging intracellular bath solution.

3.3 Results

The cells used in this study were from six consecutive passages, starting at passage 24. Cell membrane proteins were prepared from 50 flasks. Assays for the apical membrane marker alkaline phosphatase were undertaken to characterize the efficacy of the apical membrane preparation. On average, an 8 fold increase in specific activity of alkaline phophatase was measured in the apical fraction, in comparison with the activity in the basolateral fraction. A further increase in the relative amount of integral proteins in the preparation was achieved by washing the apical fraction with the alkaline buffer. This

treatment was previously shown to remove peripheral proteins (Neubig et al.1979). After this high pH buffer treatment, alkaline phosphatase activity was below detection level, with 20 % extraction of the total proteins. These proteins were subsequently used to prepare giant liposomes for patch clamp experiments.

Liposomes selected for recordings had diameters in the range 20-30 µm. Gigaohm seals between liposomes and the patch clamp electrodes were formed in about 90 % of all trials, with little or no suction applied. Control experiments were performed with liposomes made from asolectin vesicles, but in the absence of apical membranes. Using the voltage range of ±120 mV no channels activity was detected in either liposome-attached or excised, inside-out patches. The probability of finding active ion channels increased with the amount of protein used to prepare liposomes, and is shown in Fig. 3.1. Often especially at higher protein concentration, patches contained more than one ion channel.

Biophysical properties were measured for three types of anion channels, the linear 20 pS chloride channel, the outwardly rectifying chloride channel, and the large (≈390 pS) ion channel. Recordings of a chloride channel reconstituted into a giant liposome are shown in Fig. 3.2. The channel in this figure had a conductance of 18.4 pS, showed no rectification in symmetrical Cl^- solutions and was similar to the 20 pS Cl^- channel described in different native epithelia tissues (Shoemaker et al 1986; Duszyk et al 1990). The current-voltage relationships obtained with different anions in the bathing solution are shown in Fig.2.3. Membrane potential in this and other figures is reported relative to zero in the pipette. This convention has been adopted to present data in a similar manner to data from excised patches from native cells. Additionally, studies of protein orientation

after reconstitution into liposomes indicated that 75% to 95% of all proteins preserved their original orientations in cell membranes(Gennis 1989). The solid lines in Fig.3.3. represent the best fits of the GHK current equation (Eq.1) to the experimental data. The resulting permeability ratios calculated from the reversal potentials (Eq. 2) were: Cl^- 1.0(n=9), I^- 0.95±0.08(n=3), Br^- 0.71±0.05(n=4), F^- 0.59±0.05(n=6). Single-tailed Student's t-tests applied to this data indicated that each pair of mean values in the sequence were significant difference ($p<0.05$) except for the difference $I^- > Br^-$.

Recordings of outwardly rectifying chloride channels are shown in Fig.3.4. This channel had a cord conductance at negative voltages of 48.2 pS and at positive voltages of 78.5 pS. The halide permeability of this channel was different to that of 20 pS anion channel. Fig. 3.5 shows current-voltage relationships for this channel with different halide solutions in the bath. Interpolated values of the reversal potentials, with correction for liquid junction potentials were used to calculate the following permeability ratios: Cl^- 1.0 (n=7), I^- 1.34±0.06(n=4), Br^- 1.18±0.05 (n=5), F^- 0.52±0.05(n=4). T-tests for these data indicated that the permeability sequence is $I^- \geq Br^- > Cl^- > F^-$ ($p<0.05$).

Recordings of the large, 390 pS ion channel are shown in Fig.3.6 and 3.7. The channel was mostly active within the voltages of +30 mV to -30 mV, being rarely open at higher voltages. This channel had at least 5 different substates and displayed complicated kinetics with both slow and fast channel openings. Transitions between different substates were often linked to changes in channel activity, e.g. from a slow mode with openings of the order of seconds, to a fast mode when the mean open time was about 100 μs. Differences in channel conductance when Cl^- in the bath was replaced by a different halide

were within the experimental error for Cl^-, indicating that the channel is non-selective for Cl^- over other halides. The current-voltage relationships for this channel and its main substates are shown if Fig. 3.8. The channel was rarely seen fully open, being most frequently in a substate with conductance of 93 pS.

Variable mole-fraction behaviors were studied using different proportions of chloride and fluoride ions in a total of 140 mM halide. These two ions were used because they had the largest permeability differences for the 20 pS and rectifying channels. Figure 3.9 shows typical recordings of a frequently seen substate (93 pS) of a large channel in solutions having different concentrations of fluoride. The potential in pipette was 20 mV in both cases. There was no significant difference in ion channel conductance as a function of fluoride concentration. Summaries of the mole-fraction experiments for the 20 pS chloride channel, outwardly rectifying chloride channel, and the 93 pS substate of the large channel are shown in Figure 3.10. The solid lines show the predicted linear relationships if the movements of the two ions were completely independent of each other . For the large channel there was no evidence of any deviation from a linear dependence of relative current on mole-fractions. However, data for the 20 pS chloride channel and outwardly rectifying chloride channel show clear deviations from linearity.

3.4 Discussion

Three different groups of anions channels have been described in this study: the 20 pS non-rectifying anion channel, the outwardly rectifying anion channel, and the large, ~ 390 pS non-selective voltage-dependent anion channel. The existence of these channels in native epithelia has been well documented (Vaughan and French 1989; Anderson et al. 1992). The 20 pS anion channel has been shown to have a weak binding site inside the channel (about 3 kT), and its kinetic behavior could be described by a mode having three open and three closed states(Duszyk et al 1990). Recently, the halide permeability sequence for this channel was described (Wilk-Blaszak et al 1992), and anomalous mole-fraction behavior indicated that this channel probably contains a multi-ion pore. The results obtained here are in good agreement with the data from native tissues. After biochemical isolation and incorporation into giant liposomes, the channel preserved its selectivity sequence and showed similar behavior in anomalous mole-fraction experiments.

The outwardly rectifying chloride channel has been characterized recently by Halm and Frizzell (1992). These authors reported the permeability sequence $I^- > Br^- > Cl^- > F^-$, and described the channel as a multi-ion pore in which other permeate anions could affect chloride movement across the membrane. After reconstitution into liposomes, both, the permeability sequence and the variable mole-fraction data were in agreement with the results obtained in native tissues. This channel is different from the Ca^{++} dependent, rectifying anion channel described by Alton et al.(1991). The relative permeabilities of that

channel under bi-ionic conditions gave the sequence $I^- > Cl^- = Br^- >> F^-$, which is different from that reported here.

The presence of a voltage dependent, ~390 pS chloride channel in the apical membranes of epithelial cells has been documented before (Neson et al. 1984; Hanrahan et al 1985; Kolb et al. 1985; Schneider et al 1985). However, the voltage dependence of activation and inactivation is not so well understood (Vaughan and French 1989). One explanation assumes that the channels are inactivated at significantly negative membrane potentials, including the resting membrane potential, activated with depolarization around zero or above, and then inactivated with a time constant of up to several seconds. More experiments will be needed to verifying this hypothesis (Vaughan and French 1989). There are different reports about the 390 pS channel anion selectivity. Schneider et al. (1985) using pulmonary alveolar type II cells found its anion permeability sequence to be $I^- > Br^- \geq Cl^-$. Hanrahan et al. (1985) using rabbit urinary bladder epithelial cells found a selectivity sequence of $Cl^- \approx Br^- \approx I^- > F^-$. The permeability sequence described here is different from those given previously. These data suggest that although these channels have similar conductances and voltage dependence, they are not identical in different epithelia as can be judged from their halide permeability sequences.

The 390 pS chloride channel in native epithelia is rarely open, making a detailed analysis of its properties difficult. For example, in human airway epithelial cells (Duszyk et al.1989), this channel was found in 8 patches out of 495 studied (1.6%). The situation was different after reconstitution of the ion channel protein into liposomes. Within the voltages of -30 mV to +30 mV, the channel was open for more than 50 % of the time studied, and

showed at least 5 different conductance substates. Additionally, the channel showed two obvious types of kinetics, fast (opening and closing events within less than 1 ms), and slow with the openings in the order of few seconds. Often, fast kinetics events appeared superimposed on the slow kinetics (Fig. 3.6 and 3.7). The channel was not significantly selective for any of the halides, and did not show any anomalous mole-fraction behavior, indicating relatively simple permeation through the pore. the physiological role of this channel as well as its activation mechanism are unknown. However, owing to its large conductance, one might expect that activation of this channel would have a dramatic effect on intracellular ion fluxes. The kinetic properties of this channel look quite similar to the OmpF and OmpC porin channels found in the membranes of *Escherichia coli* (Berrier et al. 1992). In both cases the channels show two different kinetic modes of openings and closings, and the conductance of the porin channel (200 pS in 0.1 M KCl) is not very different from the conductance of the channels reported here. However, porins form trimers, and essentially three steps, each corresponding to about one third of the trimer conductance have been observed (Berrier et al 1992). This is clearly different from the data reported here, where at least 5 different conductance levels could be observed.

The reason for increased activation of 392 pS channel in giant liposome is unknown. One hypothesis to explain this behavior would be that the apical membrane contains inhibitor(s) of this channel which prevent its activation in cell-attached or excised inside-out modes. Another possibility is that biochemical isolation of the ion channel proteins, and especially the detergent treatment of cell membranes, could affect the ion channel protein function by chemical modification. This seems less probable, since

CHAPS is considered to be one of the gentler and less denaturing detergents (Bollag and Edelstein 1991) and also because other ion channels were not obviously affected.

More studies will be necessary to relate particular membrane proteins to observed chloride channels, but, the methods of ion channel protein isolation and incorporation into giant liposomes, described in this study, seem to be an appropriate starting point for these studies.

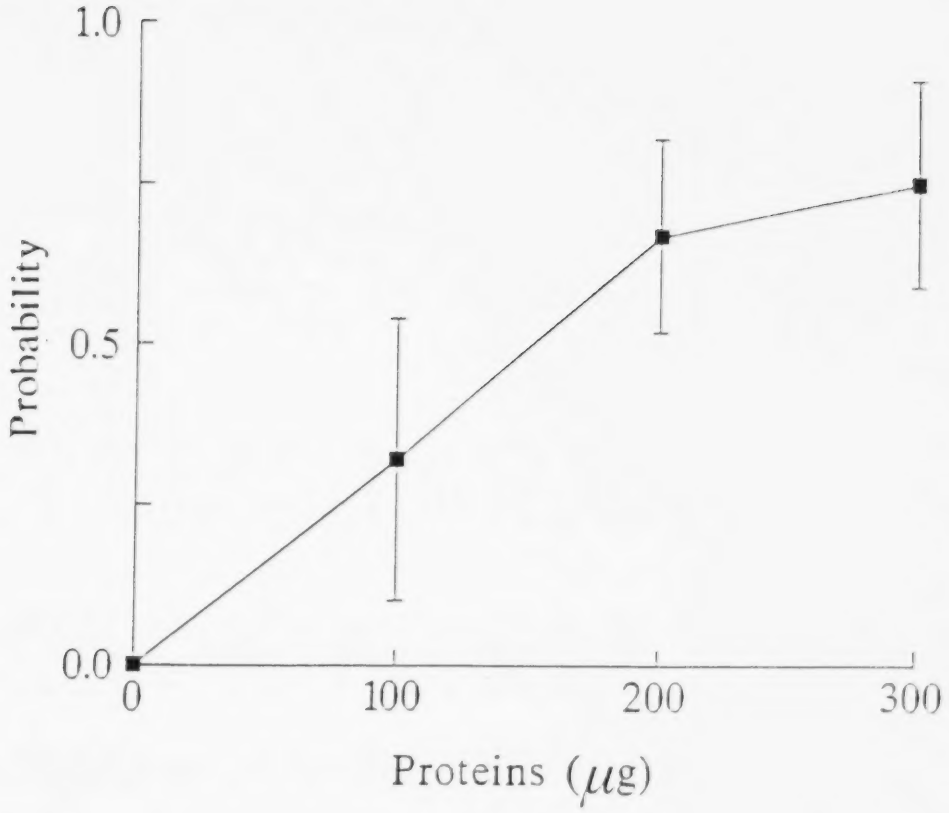

Fig. 3.1 The probability of finding an ion channel in a patch as a function of the amount of protein used to prepare the giant liposomes. The data present mean ± standard deviations from: 0 μg, 4 preparations (n=24); 100 μg, 7 preparations (n=119); 200 μg, 5 preparations (n=42); and 300 μg, 4 preparations (n=28)

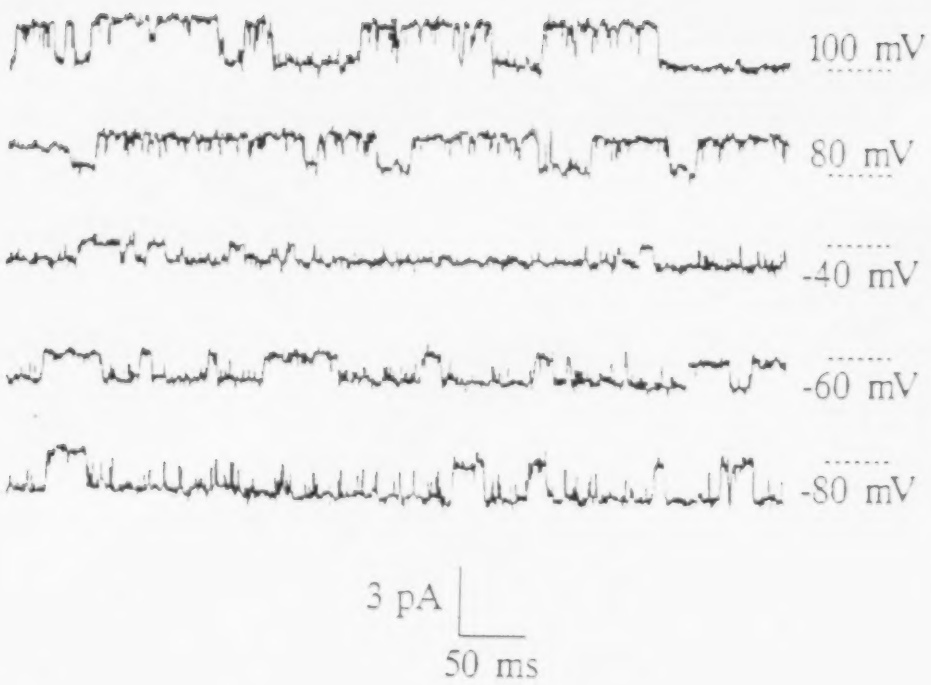

Fig. 3.2 Recordings of an anion channel in symmetric 140 mM Cl^- solutions. The channel conductance was 18.4 pS. The data were filtered at 800 Hz, and the closed state is indicated by a dashed line

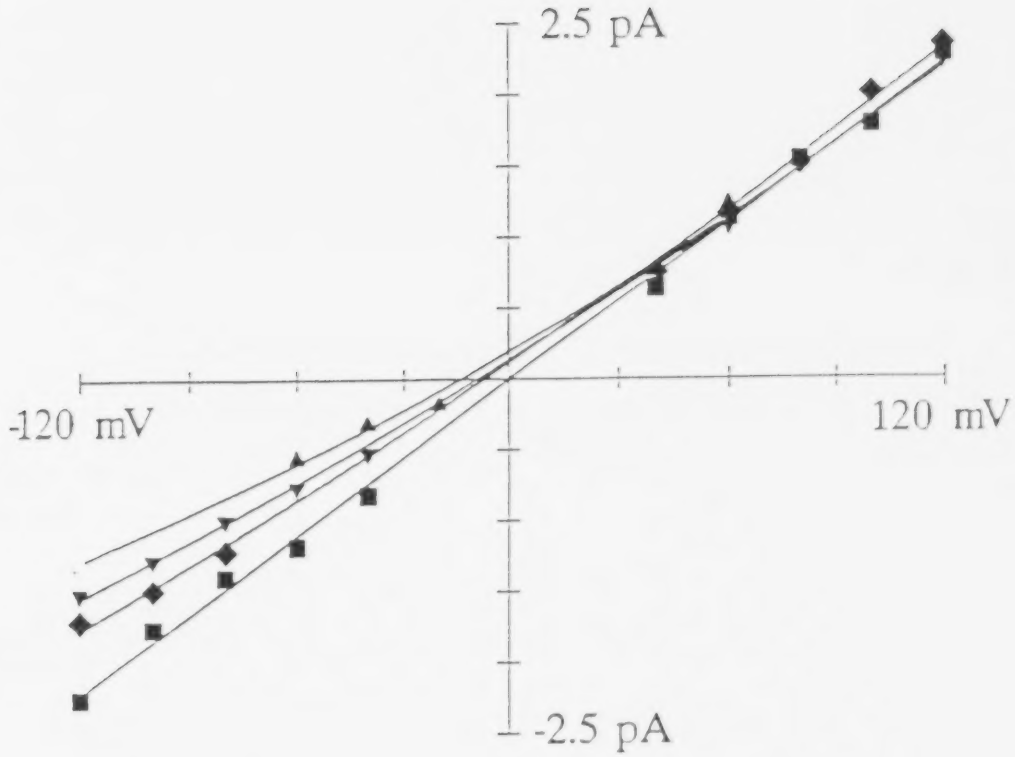

Fig. 3.3 Current-voltage relationships for a 20 pS anion channel with different halides in the bathing solution. Halide concentrations were (mM) : 140 Cl^- (squares), 140 I^- (diamonds), 140 F^- (upright triangles), 140 Br^- (inverted triangles). The pipette solution contained 140 mM Cl^-. All potentials were measured in the pipette, relative to zero in the bath

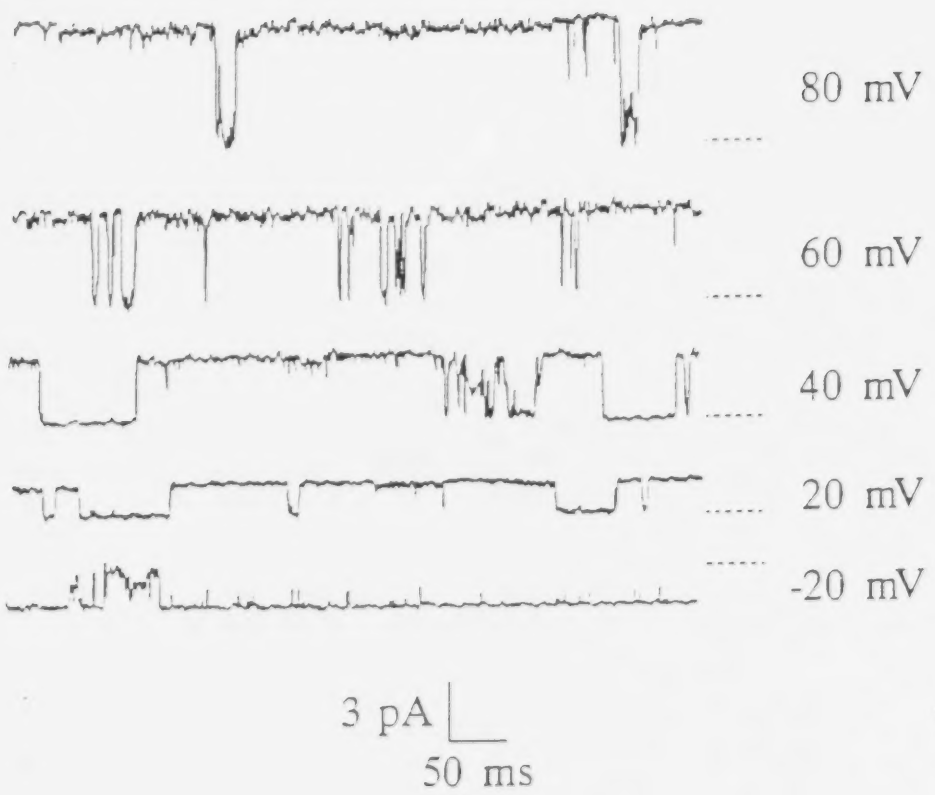

Fig. 3.4 Recordings of an outwardly rectifying anion channel. The pipette contained 140 mM Cl^- and the bath contained 140 mM Br^-. The data were filtered at 800Hz. and the closed state is indicated by a dashed line

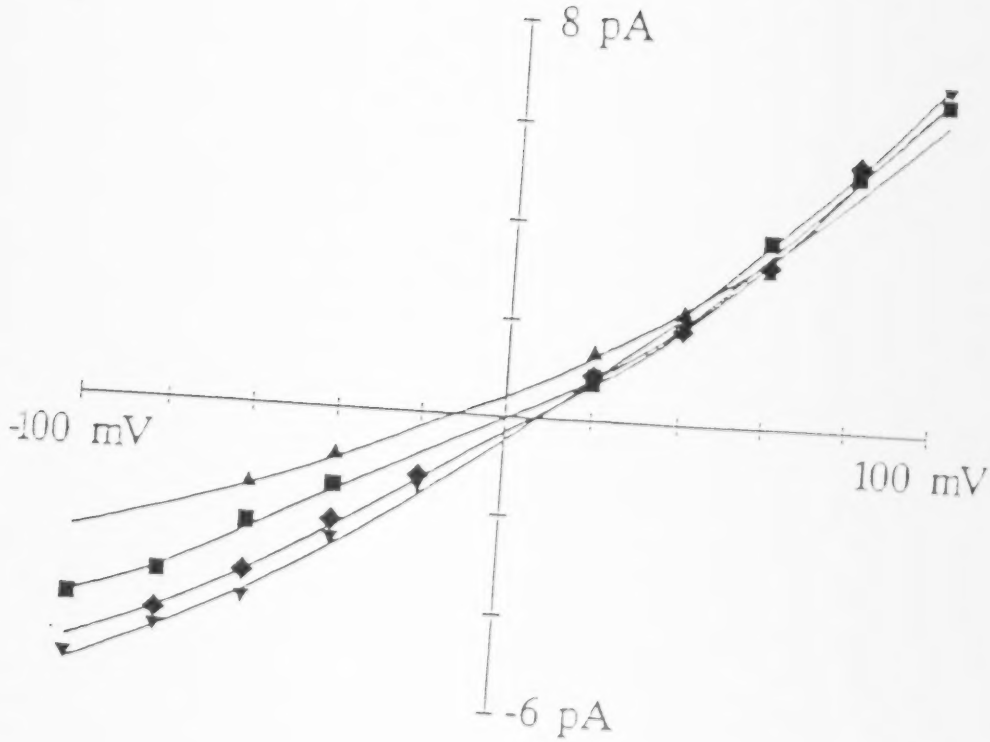

Fig. 3.5 Current-voltage relationships for an outwardly rectifying anion channel with different halides in the bath solution. Halide concentrations were (mM): 140 Cl^- (squares), 140 Br^- (diamonds), 140 F^- (upright triangles), 140 I^- (inverted triangles). The pipette solution contained 140 mM Cl^-

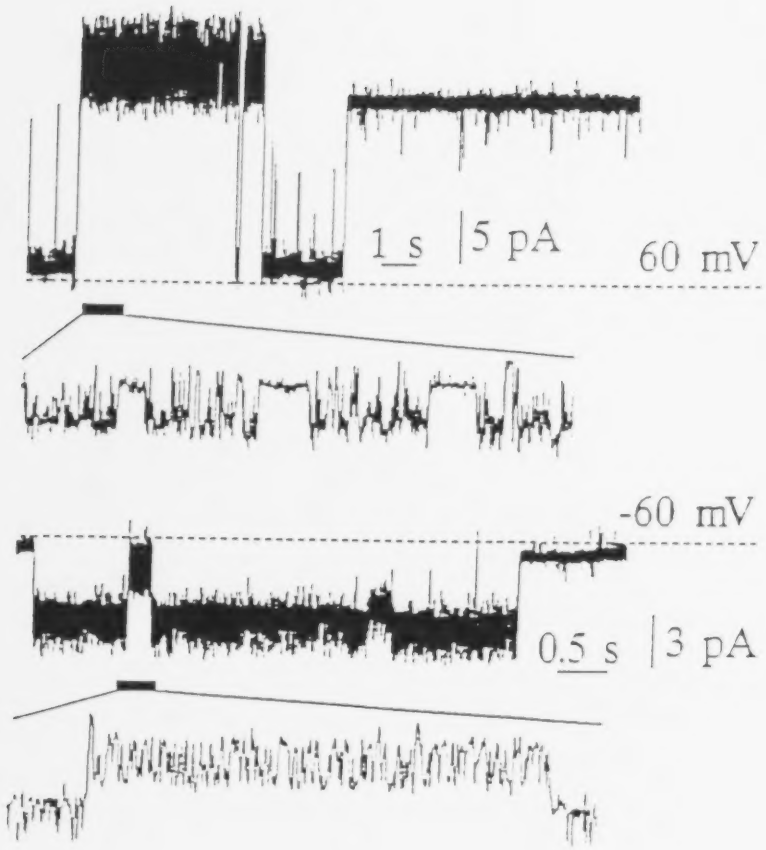

Fig. 3.6 Recordings of a large (392 pS) anion channel in symmetrical 140 mM Cl solutions. The revealed many substates and showed complicated kinetics with both slow (of the order of seconds) and fast (~1 ms) modes. The second and the fourth traces show recordings of the first and the third traces respectively (corresponding to the horizontal bars) on an expanded time scale. The data were filtered at 800 Hz, and the closed state is indicated by a dashed line

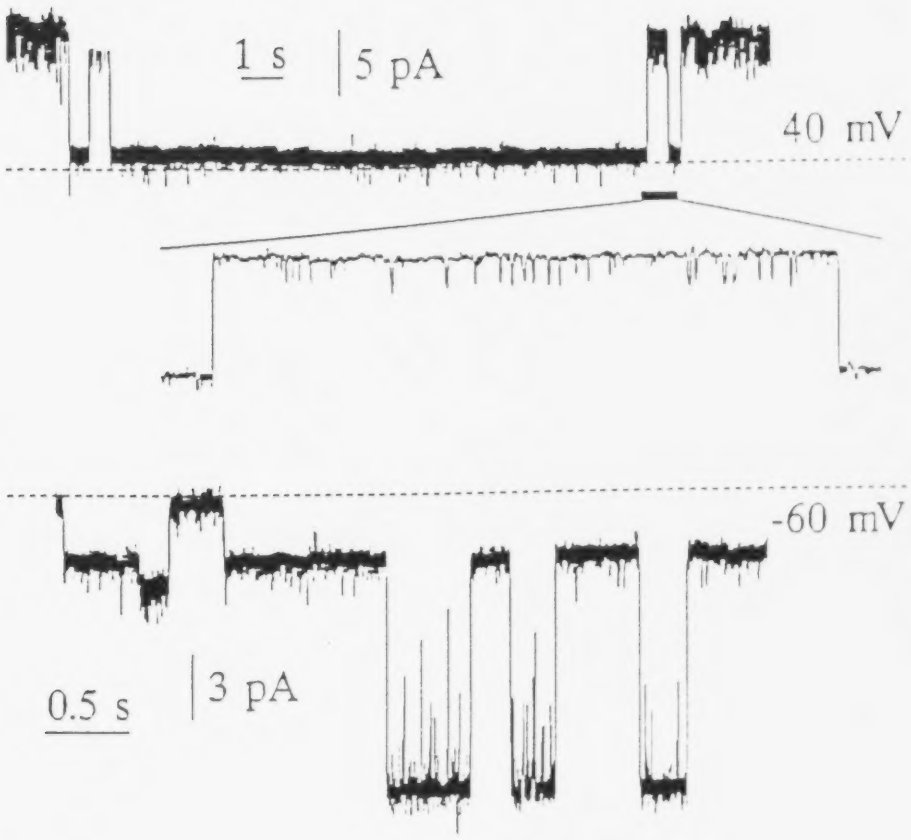

Fig. 3.7 Recordings of a large (392 pS) anion channel when the bath contained 140 mM Br^-, and the pipette contained 140 mM Cl^- solution. The middle trace shows a 1s recording of the upper trace (corresponding to the horizontal bar) on an expanded time scale. The substate shown in the expanded scale had a conductance of 52 pS. Note the fast kinetics in this substate. The data were filtered at 800 Hz. and the closed state is indicated by a dashed line

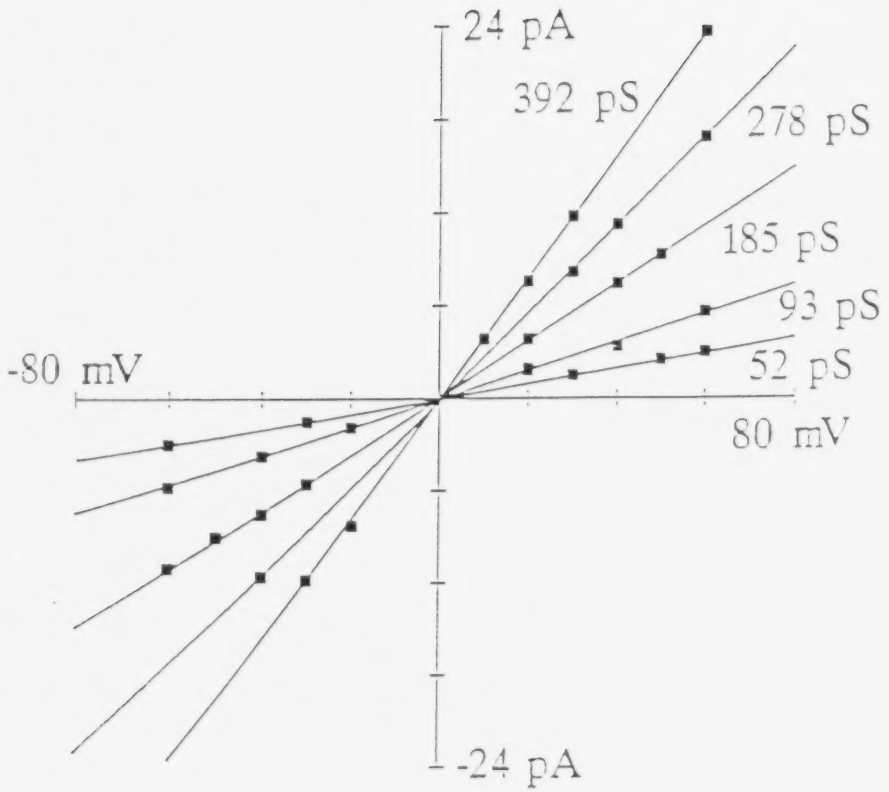

Fig. 3.8 Current-voltage relationships for the 392 pS ion channel and its major substates. Replacement of Cl^- in the bath with other halides had negligible effect on the channel or its substates conductances, indicating nonselectivity for anions

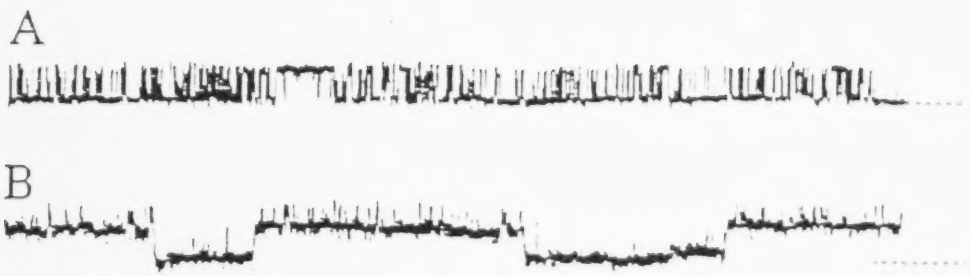

Fig. 3.9 Recordings of the most frequently seen substate (93 pS) of a large anion channel when the bath solution contained: (A) 70 mM F^- and 70 mM Cl^-, and (B) 140 mM F^-. The pipette contained 140 mM Cl^-. The voltage in the pipette was 20 mV in both cases. The data were filtered at 800 Hz, and the closed state is indicated by a dashed line

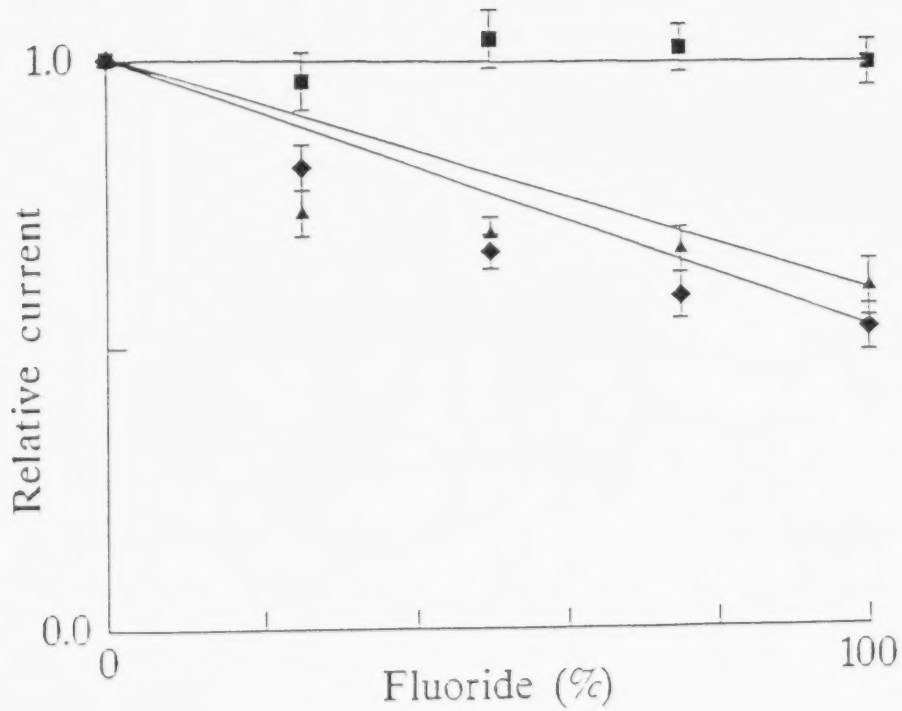

Fig. 3.10 Ion channel current at a voltage of 20 mV in the pipette, as a function of the mole-fraction of fluoride. Squares denote relative current through the major substate of the 392 pS channels, and triangles denote the relative current of the 20 pS chloride channels, and diamonds denote the relative current through the outwardly rectifying chloride channels

CHAPTER 4. CHARACTERIZATION AND REGULATION OF A CHLORIDE CHANNEL FROM BOVINE TRACHEAL EPITHELIUM*

4.1 Introduction

The regulation of apical membrane Cl^- conductance provides a crucial site for the control of salt and fluid secretion across many epithelia (Frizzell and Halm, 1990), and apical Cl^- channels provide the major control of transepithelial Cl^- movement. They are affected by many regulatory factors, including membrane potential, intracellular calcium, cell volume and protein kinases and phophatases(Frizzell and Halm, 1990).

Heterotrimeric GTP binding (G) proteins play a central role in the coupling of membrane receptors to effect mechanisms including ion channels. Ion channels are thought to be G protein effectors. Recently, nonhydrolyzable analogues of GTP, which can activate G-proteins in the absence of receptor stimulation (Brown, 1993), have been shown to affect epithelial Cl^- channels. In normal and CF airway epithelial cells, activation of G-proteins inhibited the cAMP-activated Cl^- current (Schwiebert et al. 1992), while in renal epithelium, GTPγs activated apical membrane Cl^- channels (Schwiebert et al. 1990). G-proteins inhibited the 380 pS Cl^- channels in the rat bile duct epithelial cells (McGill et al 1993), and in fetal guinea pig type II alveolar cells (Kemp et al 1993), but activated 20 pS anion channels in the apical membrane of intestinal epithelial cells (Tilly et al 1991),

* Part of this chapter has been accepted by *Journal of Physiology*

and the 5 pS Cl^- channel of rat salivary gland (Martin, 1993).

These conflicting reports of the effects of G-protein on ion channels can be reconciled by the assumption that different classes of G-protein have different actions. Mangel et al 1993 showed that the 381 pS anion channel of chinese hamster ovary (CHO) cells was activated by cholera toxin-sensitive G-proteins, and inhibited by pertusis toxin-(PTX) sensitive G-proteins. Another possibility is that the effect of a G-protein depends on the phosphorylation state of the protein, as was shown for epithelial Na channels (Isomailov et al 1994). Phosphorylated Na channels were inhibited by subsequent ADP-ribosylation with PTX, but non-phosphorylated channels were activated by PTX.

In the present study, Cl^- channels were identified from bovine tracheal epithelial cells and activation of G-proteins with NaF or GTPγs has unmasked the existence of a novel type of Cl^- channel. These channels have a conductance in the range of 100-120 pS, and are normally silent. The physiological role of these channels is uncertain. They do not constitute the majority of the Cl^- channels present in bovine trachea, since only about 25% of all Cl^- channels could be included in this group. However, the fact that they could be activated by physiologically important factors such as G-proteins, suggests that they probably play a significant role in regulating the normal Cl^- flux across the epithelium. This role would be further enhanced by their relatively high conductance.

4.2 Experimental design

The inside-out mode of patch clamp technique was employed in these experiments to characterize chloride channels from apical membrane of bovine tracheal epithelial cells. In order to determine the mechanism of the activation of NaF on a Cl^- channel, the following agents were used ; Alkaline phosphatase, GTPγS, NaF, Okadaic acid, Calyculin A, Sodium vanadate, SITS and DNDS.

4.3 Results

4.3.1 Ion channels in native bovine epithelial cells

Almost half (48%) of all successful patches (n=67) contained spontaneously active ion channels that were identified as anion selective using different Cl^- concentrations. Current-voltage relationships were determined in different NaCl solutions, and fitted by the GHK equation (Eq. 1) to obtain the channel conductance at 140 mm NaCl, and permeability ratio P_{Na}/P_{Cl}. Three groups of channels were identified, with mean conductances of 26.7 ± 3.2 pS (n=4), 68.3 ± 9.4 pS (n=25), and 108.9± 12.7 (n=3). When 140 mM NaCl in the bath was replaced by 140 NaF, the activity of 108 pS Cl^- channels increased significantly (Figure. 4.1), and 6 new channels with similar conductance could be activated in 35 silent patches. Fitting the GHK equation to the current-voltage relationships gave a mean channel conductance of 109.5 ± 11.2 pS (n-9) in 140 mM NaCl

solution, and a relative permeability ratios P_{Na}/P_{cl}= 0.04±0.03 (n=4), and P_F/P_{Cl}= .74±0.12 (n=9).

Experiments with native bovine cell were difficult to perform because of poor gigaohm seal formation. The probability of obtaining a successful seal (resistance >1 GΩ) was less than 0.08 of all attempts (n=910). On the other hand, the probability of obtaining successful seal using giant liposomes was approximately 100%. Additionally, the probability of finding a channel in a liposome could be regulated by using different amounts of proteins. Therefore, most of the experiments described in this chapter were performed with channels reconstituted into liposomes.

4.3.2 Ion channels in giant liposomes

Incorporation of 100 µg of protein from fraction A2 gave about 40% probability of finding a channel in a patch. Increasing the amount of protein increased the probability of finding a channel, but also increased the probability of finding several channels in a patch. Therefore, most liposome preparations were done with 50-100µg of apical membrane proteins.

Figure 4.2A shows recordings of a spontaneously active chloride channel reconstituted into a giant liposome. This record was obtained in the excised inside-out mode, under identical conditions to those reported for native bovine cells. The pipette contained 70 mM NaCl, and the bath 140 mM NaCl. Fiting of GHK equation (Eq.1) to these data (Figure 4.2B) gave an average channel conductance of 114.6±8.1 pS, and a permeability ratio P_{Na}/P_{Cl}=0.06±0.04 (n=11). These channels were significantly more

selective for anions than cations, could be activated by NaF and had conductances similar to those seen in native bovine tracheal cells. They were also affected by stilbene derivatives, SITS and DNDS. The probability of the channel being open as a function of transmembrane potential is showed in Figure 4.3. In symmetric NaCl solutions, the channel open probability did not depend on membrane potential (n=8), and the channels remained open about 75% of the time. Both DNDS and SITS affected channel kinetics but not channel conductance. The effect of 10 μM DNDS was independent of voltage, and caused a reduction of the open probability to 0.29±0.07 (n=5). At the same concentration, SITS was more potent in blocking channel activity than DNDS, and the effect was voltage dependent. At positive voltages the channel remained almost completely closed after application of SITS, while at negative voltages the mean open probability was 0.26±0.03 (n=8).

4.3.3 Activation by NaF

Replacement of NaCl by NaF in the bath had very pronounced effect on ion activity. NaF not only increase channel activity in the patches, it also stimulated channel activity in 35 of the total of 56 patches that were silent in NaCl solution (Figure 4.4). Activation of Cl^- channels by NaF was independent of the presence of Ca^{++}. In 6 experiments with liposomes containing 100μg of proteins from fraction A2, NaF activated Cl^- channels in 4 patches when the bath contained no free Ca^{++}.

Fluoride has been shown to affect the activity of a variety of functional proteins (Chabre, 1990). It is shown that a number of these effects are related to the fact that

fluoride forms complexes with aluminium. Trace amounts of aluminium are found naturally in biological fluids, and invariably occur as contaminants in high grade laboratory reagents or from laboratory glassware. Dr. Marek Duszyk (1995a) tested this hypothesis using the Al^{+3} chelator deferoxamine mesylate. Liposomes were prepared using 300µg of apical membrane proteins (fraction A2), and NaF activation studies were performed in the presence and absence of 2 mM of deferoxamine mesylate. In 6 of 20 patches exposed to the NaF solution alone, 100-120 pS Cl^- channels became activated. In 20 patches exposed to NaF solution in the presence of Al^{+3} chelator, no Cl^- channel became activated. These experiments indicate that the action of NaF was due to its formation of complexes with contaminant metal ions present in the solution.

The role of other membrane-bound proteins was studied by incorporating different fractions of apical membrane proteins into giant liposomes. One such experiment involved the removal of membrane-bound proteins from fraction A2 by alkalization (Neubig et al. 1979). This procedure removed alkaline phosphatase from the fraction, so that its activity was below the detection level. After this treatment, ion channel activity in patches excised from giant liposomes was retained, as 5 spontaneously active Cl^- channels with conductance 100-120 pS were observed, But NaF did not affect the activity of these channels. Additionally, in 21 patches exposed to NaF solution no Cl^- channels became activated. Alkalization results suggested the involvement of membrane-bound proteins in ion channel activation by NaF. Since fluoride has been shown to interfere with a large variety of enzymes including adenylate cyclase, phopholipase A_2, ATPases, phophatases

and G-proteins (Brown, 1993), limited experiments were designed to identify the pathway(s) of chloride channel activation by NaF.

4.3.4 Effects of protein phophatases on ion channel activity

NaF could possibly activate ion channels by inhibiting protein phophatases, which close ion channels by dephosphorylation. This mechanism could be particularly important because apical membranes contain a significant amount of membrane bound AP. To test this hypothesis, 20 U/ml of bovine AP was applied to patches containing spontaneously active Cl^- channels (n=6), or channels activated by NaF (n=5). An example of such an experiment is shown in Figure 4.5A. AP did not affect ion channel activity in the voltage range ± 100 mV. The role of other protein phosphatases was investigated by using the specific inhibitors calyculin A and okadiac acid. These inhibitors were applied in the presence of mM of ATP in the bath. If these channels were regulated by the phosphrylation/dephophorylation cycle, then inhibition of phophatases activity could facilitate channel opening by protein phosphorylation. Neither 10 μM calyculin a (n=6) nor 10 μM okadiac acid (n=9) could activate ion channels in silent patches. Okadiac acid had no effect on ion channel activation in this silent patch (Figure 4.5B, middle tracing), although the presence of a channel in the patch was subsequently shown by replacing the bath solution with NaF (Figure 4.5B, bottom tracing). Application of NaF to silent patches after exposure to okadiac acid activated 3 channels with conductance 100-120 pS, and 2 channels after exposure to calyculine A. Similar experiments were performed with sodium vanadate, an inhibitor of AP. In twelve such experiments, no channel activation was

observed after application of 1 mM sodium vanadate and 1 mM ATP, while channels were subsequently activated by NaF in five patches.

4.3.5 Effects of G-proteins on ion channel activity

To test the hypothesis that G-proteins could activate Cl^- channels, we used a non-hydrolysable analog of GTP, guanosine 5'-[γ-thio]triphosphate (GTP γS) which is a stimulator of G-proteins. Excised, silent patches were exposed to 100 μM of GTPγS in the bath solution. In 7 of 26 patches studied, channel activity appeared within 5 min after adding GTPγS (Figure 4.6). Both the mean channel conductance, 107.6±9.1 pS, and the mean open channel probability, 0.67±.12, were similar to those of NaF activated channels. Patches that were silent in the presence of GTPγS were subsequently exposed to 70 mM NaF solution, and no 100-120 pS channels were seen, indicating their absence in these patches. Since stimulation of Cl^- channels did not require the presence of ATP in the bath, these experiments indicate a direct activation of Cl^- channels by G-proteins.

4.4 Discussion

Ion channels are often reconstituted into artificial membranes to investigate their biophysical properties. One potential problem with this approach is that the function of a channel may be altered as a result of separation from the native membrane, or incorporation into the new membrane. Kapica et al. (1994) studied changes in channel properties during reconstitution of Ca^{++}-activated K^+ channel into lipid bilayers, and found that a channel in either situations had similar conductance, voltage dependence, activation

by intracellular Ca^{++} and sensitivity to extracellular blockers, such as tetraethylammonium (TEA) and charybdotoxin. However, channels in artificial bilayers required five-fold higher free Ca^{++} for activation, and the TEA block was less voltage dependent. Other studies showed that outwardly rectifying epithelial Cl^- channels preserved their function and regulatory mechanisms after reconstitution in to lipid bilayers (Bridges & Benos, 1990). It seems clear that if the cytoskeleton, or some cytoplasmic enzymes were involved in channel regulation, then channel function could be altered by reconstitution into artificial membranes. Therefore, we cannot be certain that the reconstituted channels were identical to those seen in native cells. However, the facts that they had similar conductances, P_{Na}/P_{Cl} and P_I/P_{Cl} permeability ratios, and were activated by NaF, strongly suggest that they retained their major functions after reconstitution into liposomes.

The main criterion used for including a Cl^- channel into one of the classes described here, was its sensitivity to GTPγS and / or NaF. Channels could be activated by these chemicals in silent excised patches, or could increase their activity in already active patches. There was significant variability in the gating of these channels (Figures 4.2, 4.4, and 4.5), as well as in their single channel conductance (92 pS to 147 pS). Similarly, some channel recordings contained distinct substates in both native cells and from liposomes (Figures. 4.1A, 4.2A and 4.6A). However, these data include results from a significant number of different bovine tracheas, and these variabilities could reflect natural differences between G-protein-sensitive Cl^- channels from different animals.

4.4.1 Channel block

There are numerous reports on the effects of stilbene dissulphonates on ion channel function. 4,4'-bis(isothiocyano)-2,2'-stilbenedisulfonate (H_2DIDS) has been reported to completely inhibit 25-30 pS bovine tracheal Cl^- channels reconstituted into lipid bilayers (Ran et al. 1992). Inhibition of rat colonic Cl^- channels by DNDS was reversible, while inhibition by DIDS was irreversible (Bridges and Benos, 1990). SITS is a well-known representative of a large variety of reagents that react rather specifically with either the ε-amino groups of lysine residues, or the amino group at the N-terminal end of a protein (Means and Feeney, 1971). SITS conferred a voltage sensitivity to channel gating without affecting conductance properties. It is possible that the channel protein became covalently modified, as the effect of SITS was irreversible. Covalent modification of proteins is an important factor in regulation of enzyme activity (Means and Feeney, 1971). Recently, it has been shown that direct covalent modification of the epithelial Na^+ channel protein by protein kinase A conferred similar voltage sensitivity to channel gating (Ismailov et al. 1994). The effect of SITS suggests a crucial role for either lysine residues or the N-terminal amino group in the process of ion permeation. This prediction cannot be verified until the amino acid sequence of this bovine epithelial channel and its conductive pathway are known.

4.4.2 Activation by NaF

Studies in the cell-attached mode indicated that this channel was silent under non-stimulating conditions. Excision of the patch from the membrane had little effect on its

activity, indicating that cytoskeleton-channel protein interations do not affect channel activity. However, channels were often activated when chloride in the bath solution was replaced by fluoride. The most straightforward explanation of this phenomenon would be that F^- ion interact directly with the channel protein, changing its conformation and leading to channel activation. However, earlier studies (Collins and Washabaugh, 1985) showed that fluoride is a kosmotropic substance (polar water-structure maker) that stabilizes proteins, and therefore should not affect ion channel activity.

It is well-known that fluoride interacts directly with a number of enzymes, including ATPases, phspholipases, phsphatases and G-proteins (Brown, 1993). The actions of fluoride are due to flouride forming complexes with traces of aluminum or beryllium. The active species is thought to be AlF_4.

Although aluminum ions were not added to our solutions, we could not excluded the possibility of their presence, considering the relative impure preparation of commercial NaF. Additionally, most cell-culture media are contaminated with aluminum, and glass pipettes can be another source of contamination, since aluminum is a normal component of glass, and can be etched by fluoride solutions. Therefore, aluminum ions are often present in salt solutions at micromolar concentrations, sufficient to form active AlF_4 complexes, as was shown in experiments by Dr. Marek Duszyk with the Al^{3+} chelator, deferoxamine mesylate. NaF was unable to activate Cl^- channels in the presence of the aluminium chelator, indicating its requirement for Al^{3+} ions. The relatively high concentration of NaF that we used in the experiments could also have its own effect, for example by binding Ca^{2+} ions to form CaF_n complexes analogous to those of aluminium.

However, the activation of Cl^- channels by fluoride was independent of Ca^{2+} concentration in the bath. There are also reports that the CHAPS used in the preparation of membrane proteins can have an effect on ion channel function (Breitwieser, 1991). Therefore, in some experiments another detergent, octyl β-D-glucopyranoside was used. NaF could activate Cl^- channels extracted from cell membranes using either CHAPS or β-D-glucopyranoside. Similarly, there were no significant differences in the probability of finding a channel in the patch, suggesting that both detergents interact in the same way with the channel protein.

4.4.3 Membrane-bound proteins

Patch clamp studies have shown that many patches containing ion channels also contain membrane-bound protein phosphates, protein kinases and other regulatory enzymes (Bielefeldt and Jackson, 1994). Membrane-bound proteins have also been shown to incorporate into lipid bilayers along with ion channels in several studies (Ildefonse and Bennett, 1991). In this study, incorporation of the total fraction of apical membrane proteins produced channels indistinguishable from those seen in native bovine airway epithelial cells, indicating that after incorporation into liposomes, solubilized ion channel proteins achieved their original configuration with respect to other apical membrane proteins. Removal of membrane-bound proteins significantly affected chloride channel activation. In particular, the lack of Cl^- channel activation by NaF indicated the possible involvement of protein phosphatases, G-proteins, phospholipases or their combinations in ion channel regulation. These results suggest that channel proteins form functional complexes with other membrane-bound proteins. This hypothesis is supported by the

experiments of Hamilton et al. (1991) who found that some membrane-bound proteins co-purified with Ca^{2+} channels and were co-precipitated when the channels were immunoprecipitated with a specific Ca^{2+} channel antibody. Similarly, epithelial Na^{+} channels were shown (Ausiello et al.1992) to form a 700-kDa protein complex that produced six major protein bands on reducing sodium dodecyl sulphate (SDS) polyacrylamide gels. Consequently, ion channels may form complexes with regulatory membrane-bound proteins, suggesting modular organization within the cell membrane.

4.4.4 Protein phosphatases

Alkaline phosphatase is a membrane-bound enzyme that is present in significant amounts in the apical membrane. Its role in airway epithelial cells is unknown, and it could control ion channel activity by dephosphorylation. This hypothesis is supported by the observations that when membrane-bound proteins were removed by alkalization or apical membrane proteins were purified using WGA chromatography (Duszyk et al 1995a), NaF had no effect on channel activity. However, the 100-120 pS chloride channels described here were insensitive to AP, and another inhibitor of AP, sodium vanadate, had no effect on ion channel activity. Therefore, AP is probably not involved in the regulation of these Cl^{-} channels.

The involvement of other protein phophatases, was tested by using their specific inhibitors, okadaic acid and calyculin A. Since neither drug affected Cl^{-} channel activity in patches from native cells or from giant liposomes ,these phosphatases are probably not involved in ion channel regulation. The fact that NaF could activate ion channels in the

absence of ATP is also inconsistent with the involvement of a phosphorylation-dephosphorylation cycle. NaF could inhibite AP, or the dephosphorylation process, but phosphorylation would be necessary to open the channel. Even if protein kinase A were present in the patch, ATP would still be necessary to open the channel. Therefore, it could be concluded that protein phosphatases do not play a significant role in the regulation of this channel.

4.4.5 Phospholipase A_2 (study by Dr. Duszyk)

An alternative possibility is that NaF could open ion channels by activating the membrane-bound enzyme, phospholipase A_2 (Brown), 1993). Arachidonic acid produced by phospholipase A_2 from phospholipid is metabolized to compounds which may act as second messages regulating ion channels. Such a regulatory pathway was shown previously for Cl^- channels in bovine chromaffin cells (Doroshenko, 1991). However, experiments by Dr. Duszyk (1995a) have shown no evidence to indicate the involvement of phospholipase A_2 in the regulation of airway epithelial cells. The phospholipases A_2 blocker, quinacrine, had no effect on ion channel activation either by GTPγS, or by NaF. Therefore we can probably rule out the involvement of phospholipase A_2 or its products in the regulation of these channels.

4.4.6 G-proteins

G-proteins are a family of heterotrimetric proteins that couple plasma membrane receptors to a variety of effectors including ion channels (Brown, 1993). G-proteins affect channel function either directly by membrane-delimited pathways, or indirectly by

cytoplasmic pathways involving second messages and protein kinases (Schwiebert et al. 1990). Membrane-delimited regulation of chloride channels has been characterized in several epithelial tissues (Schwiebert et al.1992; Mangel et al. 1993; Kemp et al. 1993; McGill et al. 1993; Tilly et al. 1991).

In this study, experiments suggest that the 100-120 pS Cl^- channels of bovine tracheal epithelial cells fulfill the criteria for membrane-delimited regulation by a G-protein system. They were activated by GTPγS or by NaF, without the presence of ATP. Removal of membrane-bound proteins deactivated the Cl^- channels, indicating a crucial role in channel regulation.

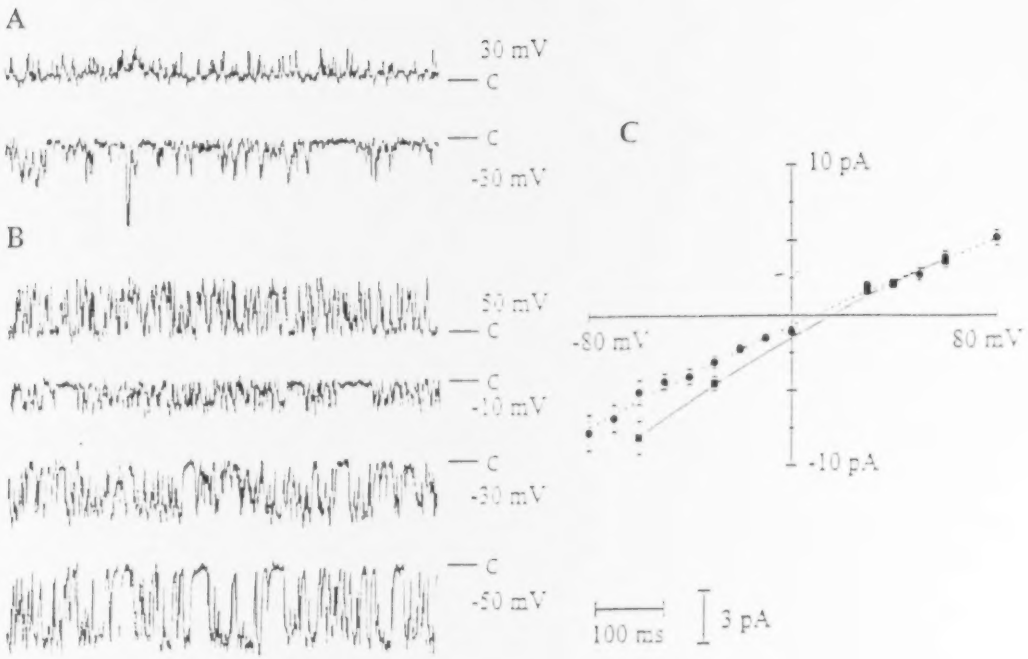

Fig. 4.1 Cl^- channel from a bovine tracheal epithelial cell. Excised single channel recordings. A: The pipette contained 70 mM NaCl, and the bath 140 mM NaCl: B: The same patch in 140 mM NaF solution. The closed state is indicated by the letter C in each case. C: Current-voltage relationships for the records shown in panels A (squares) and B (circles). The data are presented as the mean ± SD of the single channel current measurements. The lines through the data were obtained from equation (1). The solid line shows the best fit of the GHK equation under non-symmetrical NaCl conditions and gives a channel conductance of 124.5 pS, and a permeability ratio of $P_{Na}/P_{Cl} = 0.04$. Fitting the GHK equation to the data when NaCl in the bath was replaced by 140 mM NaF (dashed line), gave a permeability ratio of $P_F/P_{Cl} = 0.74 \pm 0.03$

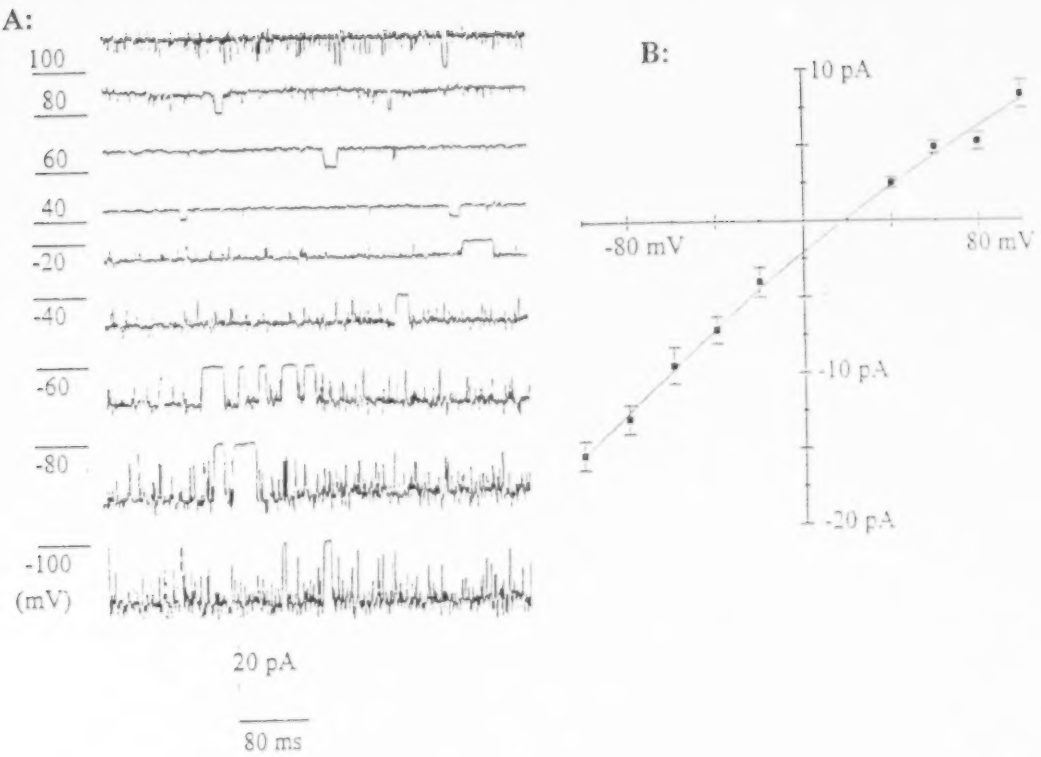

Fig. 4.2 Cl^- channel reconstituted into giant liposome. A: Excised single channel recordings. The pipette contained 70 mM NaCl, and the bath 140 mM NaCl. The closed state is indicated by a solid line. B: Current-voltage relationship for the record shown in panel A. The solid line indicates the fit of the GHK equation (1) to the current-voltage data. The channel was significantly more selective for anions than cations $P_{Na}/P_{Cl}=0.05$, and had a conductance of 112.7 pS in 140 mM NaCl solution. The data are shown as mean ± SD of the single channel current measurements

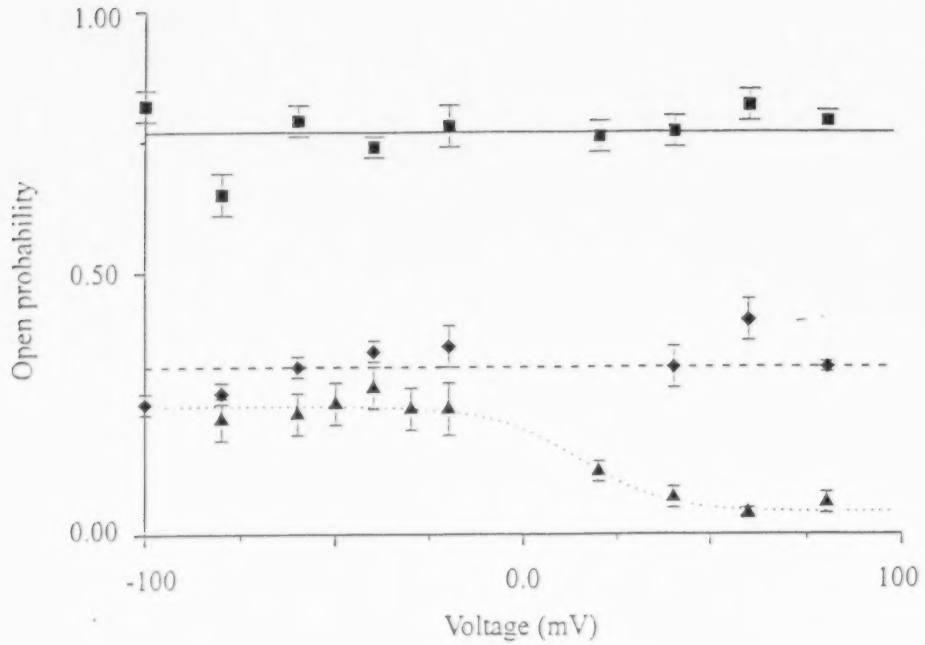

Fig. 4.3 Probability of the channel being open as function of membrane potential. Squares - symmetrical 140 mM NaCl solution (n=28), diamonds - 10μM DNDS (n=5), upright triangles - 10 μM SITS (n=9). In symmetrical NaCl solution, the channel open probability did not depend significantly on membrane potential, and was 0.77 ± 0.05 (solid line). DNDS reduced mean open probability to 0.32 ± 0.05 (dashed line). SITS caused voltage-dependent block, being most potent at positive voltages. The dotted line was drawn by eye. The data are shown as mean ± standard error.

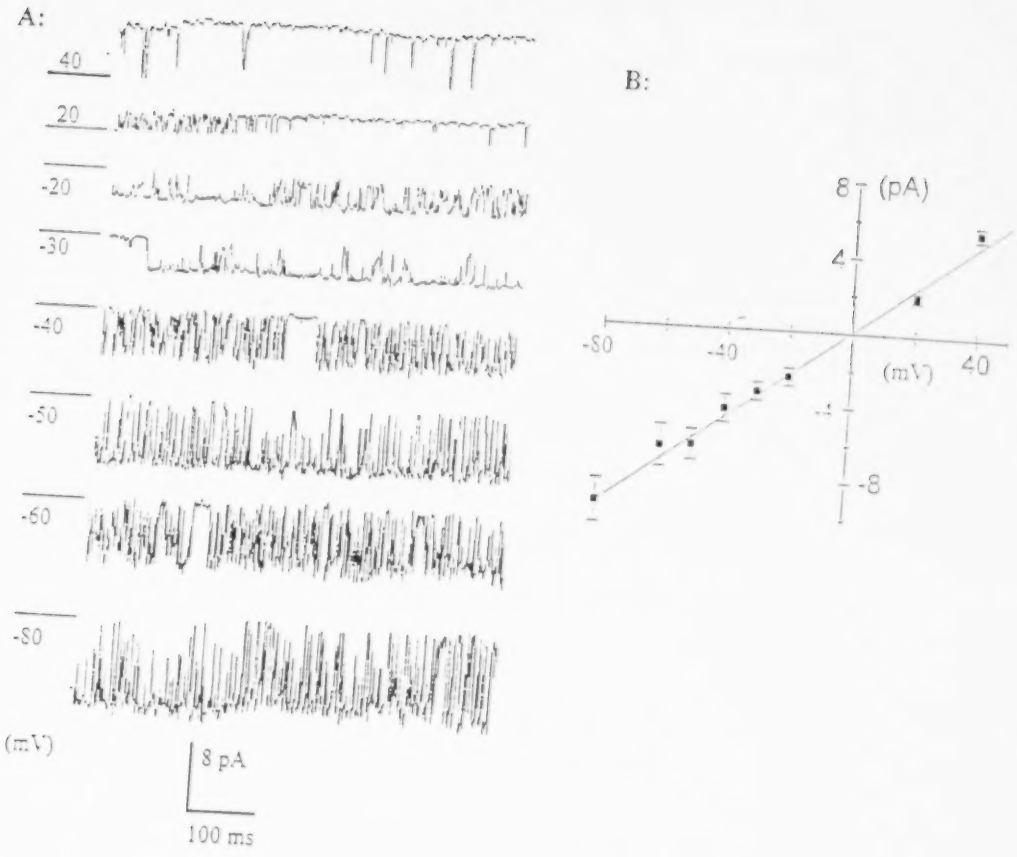

Fig. 4.4 NaF activation of a Cl^- channel. A: Liposome used in the experiments contained from fraction A2. A silent channel in symmetrical 140 mM NaCl became active when 70 mM NaCl in the bath was replaced by 70 mM NaF. B: Recordings shown in panel A were fitted with the GHK equation (solid line). This channel had a conductance of 119.2 pS in 140 mM NaCl, and P_{Na}/P_{Cl}=0.97.

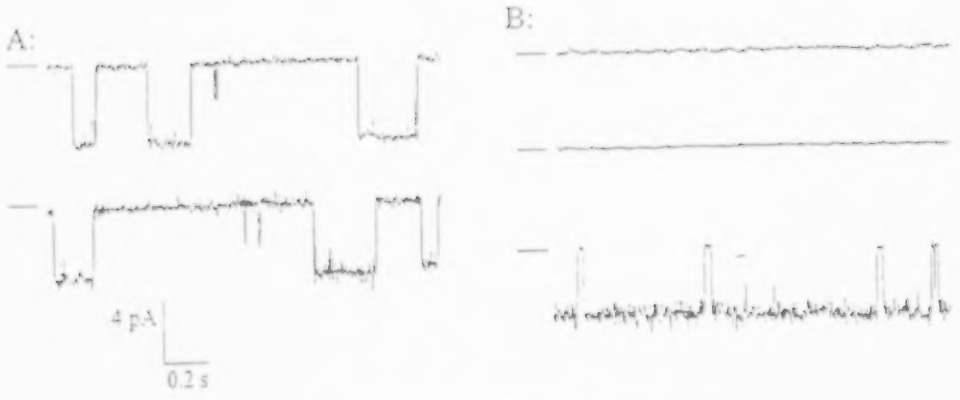

Fig. 4.5 Lack of effects of AP and okadaic acid on ion channel activity. A; The upper tracing shows a channel in symmetrical 140 mM NaCl solution. In the lower tracing, 20 U/ml of bovine AP was added to the bath. B: The upper tracing shows a recording from a silent patch. Middle tracing - 10 μM of okadaic acid and 1 mM ATP were added to the bath. The bottom tracing shows the presence of a channel in this patch by washing the bath with NaF. Recordings in both panels were at -40 mV. The data were filtered at 800 Hz, and the closed state is indicated by a solid line.

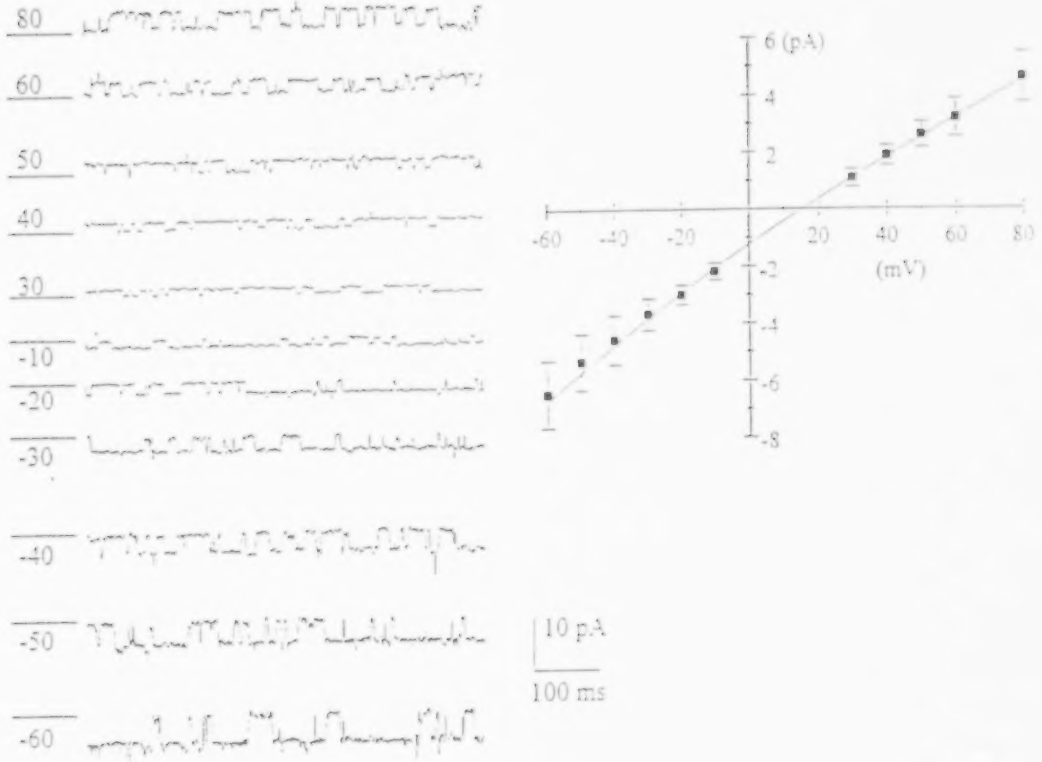

Fig. 4.6 Activation of Cl^- channel by GTP-γS. A: The liposome used in the experiments contained proteins from fraction A2. A silent channel become active when 0.1 mM GTP-γS was added to the bath. The pipette contained 70 mM NaCl and the bath 140 mM NaCl. B: Recordings shown in panel A were fitted with GHK equation (1) (solid line). This channel had a conductance of 104.3 pS in 140 mM NaCl, and $P_{Na}/P_{Cl}=0.04$

CHAPTER 5. EVIDENCE THAT pH-TITRATABLE GROUPS CONTROL THE ACTIVITY OF A LARGE EPITHELIAL CHLORIDE CHANNEL*

5.1 Introduction

Several groups of different ion channels have been identified in the apical membrane of epithelial cells, including a large conductance (340-450 pS) anion channel (Kemp et al. 1993, Xu and Lu 1994). Most investigators reported that the open probability of the large channels is voltage sensitive with activation of the channels at the voltages around -10 mV to +10 mV. However, the voltage dependence of activation and inactivation is not well understood(Vaughan and French 1989). Although the physiological role of these channels is not clear, several studies indicate that they are regulated by endogenous GTP-binding proteins, and may be an integral part of transepithelial fluid secretion in the lung (Kemp et al.1993).

The porpose of the present research was to examine the molecular nature of a large Cl^- channel's gating mechanism by focusing the possible existence of titratable charges or other fixed charges on the channel protein in a giant liposome. The behavior of a large conductance Cl^- channel during the changes of bath solution pH were examined to detemine if different pH altered the gating mechanism and activity of a large epithelial Cl^-

* Part of this chapter has been accepted for publication by *Biochemical & Biophysical Research Communication.*

channel.

5.2 Experimental design

The channels isolated from bovine tracheal epithelial cells were incorporated into giant liposome suitable for single channel recordings as discribed in earlier chapters. Experiments were performed in the excised, inside-out mode. The changes of pH in bath solution was monitored with a micro pH electrode (Model PHR-146, Whatman) during the recordings. Several parameters were calculated to determine if the change in pH altered the large conductance Cl^- channel nature; the channel open probability P_o, the steepness of the voltage dependence N_z, the voltage at which half of the channels are open V_o, and the number of proton binding sites N_h.

5.3 RESULTS

Figure 5.1A shows single channel recordings from a large conductance epithelial Cl^- channel at different bath pH values. The mean conductance for this type of channel was 396±27 pS (n=19), and was unaffected by pH changes in the bath solution (Figure. 5.1B). The channel was significantly more selective for anions than cations. When 140 mM NaCl in the bath solution was replaced by 70 mM NaCl, fitting the Goldman-Hodgkin-Katz (GHK) current equation (Hille, E. 1992) to the current-voltage relationship gave a permeability ratio P_{Na}/P_{Cl} =0.11±0.03 (n=4). Similar P_{Na}/P_{Cl} permeability ratios were

obtained at pH 6.5, 7.4 and 9.2. At each pH studied, the channel was more active at depolarizing than at hyperpolarizing voltages.

The channel activity was examined with different pH in bath solutions. Alkaline pH in the range 7.4-9.2 had no appreciable effect on channel conductance and gating. However, acidic pH significantly reduced channel open probability . This effect could be caused by stabilization of channel closed states, or by destabilization of channel open states. The effects of low pH on channel behavior suggested that the primary effect of protons is on channel gating. Figure. 5.2 presents the effects of both voltage V_m and pH on the channel open probability P_o. The data have been fitted by a Boltzmann relationship of the form:

$$P_o = \frac{1}{1+\exp(-N_Z F(V-V_o)/RT} \qquad (1)$$

where N_z is a parameter describing the number of charges which traverse the membrane during the transition between the open and closed states, V is the voltage, V_o is the voltage at which the probability of a channel being open or closed are equal , F, R, and *T* are the Faraday constant, the gas constant and the temperature, respectively. N_z is a measure of the steepness of the voltage dependence. If N_z is 0, then the channel is voltage independent. The free energy difference between channel open and closed states ΔG is the sum of voltage-independence W, and voltage-dependence components, N_zFV ($\Delta G=W+N_zFV$). When $V=V_0$, $\Delta G=0$ and $W=-N_zFV_o$. Changes in the parameters N_z and V_o as a function of pH can therefore reflect changes in the voltage-independent channel free energy component.

The fitted parameters, V_o, N_z, and P_o were determined for different pH values. N_z was not significantly affected by pH variations, and the best fit was obtained for $N_z = 0.78 \pm 0.15$. This indicates that titration did NOT affect channel voltage gate. V_o changed from -48.36 mV at pH=9.2, to -2.72 mV at 3.5. This change suggests that, in the absence of an electric field, the free energy difference between channel open and closed states decreased with an increase in bath pH. P_o decreased while pH decreased from 7.4-3.5, indicating proton reduced the activity of the Cl^- channel.

The parameters N_h, the number of proton inhibitory sites and pK_a were evaluated from the equations worked by Dr. Marek Duszyk (1995b): $N_h = 1.27 \pm 0.34$ and $pK_a = 6.09 \pm 0.42$, respectively.

5.4 Discussion

There are several earlier reports on the effects of pH on Cl channel function. Hanke and Miller (1993) found that the opening probability of the Cl^- channel from the *Torpedo electroplax* membrane increased significantly as the pH was lowered. The authors suggested that some basic residues were involved in modulating ion channel activity. Bowen et al. (1993) studied the outer mitochodrial membrane channel VDAC, and found that decreasing pH reduced the steepness of the voltage dependence N_z, while simultaneously increasing the voltage, V_o, was needed to induce the transition. However, the voltage-independent energy difference between the states, $W = N_z F V_o$, remained

relatively constant, suggesting that increasing pH neutralized the channel voltage sensor. The authors concluded that a group of lysine residues probably formed a portion of the channel gate, sensing changes in the electric field.

In this study, N_z was calculated in different pH bath solutions. Parameter N_z can be interpreted as a measure of the minimum number of charged groups on the protein which detect the applied electric field and allow the protein to respond to the field. A decrease in this parameter indicates neutralization of some of these charges, and a reduction in the channel's ability to respond to the electric field. The fact that N_z did not change appreciably with pH suggests that pH does not affect the voltage dependence of the gating reaction.

The effects of low pH on channel behavior suggested that the primary effect of protons was on channel gating, reducing channel open probability (Fig. 1). Dr. Duszyk has modified Hill'equation and estimated the number of H^+ binding sites. Let a channel protein has N_h acidic groups X that can bind protons according to the reaction,

$$X + N_h H^+ \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} XH^+_{N_h} \tag{2}$$

Then, the fraction of X in the unprotonated form is given by:

$$\frac{X}{X + XH^+_{N_h}} = \left(1 + 10^{(pK_a - N_h pH)}\right)^{-1} \tag{3}$$

where $pK = -log(k_{-1}/k_1)$.

If we assume that P_o is proportional to the number of groups in the unprotonated form, then:

$$P_o = f(V)(1+10^{(pK_a - N_h pH)})^{-1} \quad (4)$$

In the general case, if a pH-independent component of P_o, called P_{in} exists, then P_o is given by:

$$P_o / P_{max} = P_{in} / P_{max} + f(V)\left(1 - P_{in} / P_{max}\right)\left(1+10^{(pK_a - N_h pH)}\right)^{-1} \quad (5)$$

where P_{max} is the maximal value of P_o.

The fitted parameter N_h was not significantly affected by pH or voltage changes, and was equal to 1.27± 0.34. This value is not significantly different from one, indicating that there is probably a single proton binding site on the channel protein. However, since N_h gives a minimum estimate of binding sites, we cannot exclude the possibility that other sites with lower affinities are also involved.

Most of the changes in channel activity were measured while the pH was decreasing from 7.4 to 3.5. Therefore, the possible identities of the gating charges associated with the channel protein can be limited to the side chains of amino acids such as histidine, aspartic acid, glutamic acid, and the terminal carboxyl groups. The intrinsic pK_a values of these groups within proteins have been reported to be 5.2-6.4, 3.0-4.7, 4.3-4.5, and 3.5-4.3, respectively (Creighton, 1993). The parameter pK_a was evaluated by fitting Equation (5), giving mean value of pK_a was 6.09±0.42. This value of pK indicates that histidine or some acidic amino acids could probably be involved in the pH modulation of channel activity.

In this study, the results show that the large epithelial Cl^- channel has at least one titratable site located on the intracellular site of the membrane that can be modified by

changes in solution pH. The fact that channel conductance is pH insensitive, indicates that the protonation site is probably not close to the channel permeation pathway. If the titratable groups were located within the channel pore, then one would expect that the protonation-deprotonation reaction would lead to a flickering current signal in single channel recordings (Hille, 1992). Alternatively, if the protonation reaction were too fast to be resolved in patch-clamp experiments, one would expect a decrease in channel current, as was observed in L-type Ca^{++} channels (Prod'hom, et al 1987). If the protonation site were located near the channel entrance, then changes in electrostatic interactions caused by pH would affect the distribution of ions near the channel pore, and therefore its measured conductance. However, no significant change in channel conductance was observed (Fig. 1). These results also indicate that both the conduction pathway and the channel entrance are electrostatically isolated from the charged head groups on the membrane lipids, because these would also be titrated by the pH changes.

Large-conductance anion-selective channels have been described in a variety of cell types but their physiological function is unknown. It is possible that these channels play a transient role in internal ion regulation in cells, or in intracellular pH regulation by being permeable to HCO_3^- ions. However, their relatively great abundance in a large number of cell types, and regulation by physiologically important enzymes such as G-proteins, suggests that they are important in cell physiology. These channels will normally experience a significant variation in the pH of their environment, so that knowledge of their pH sensitivity is crucial to understanding their normal function.

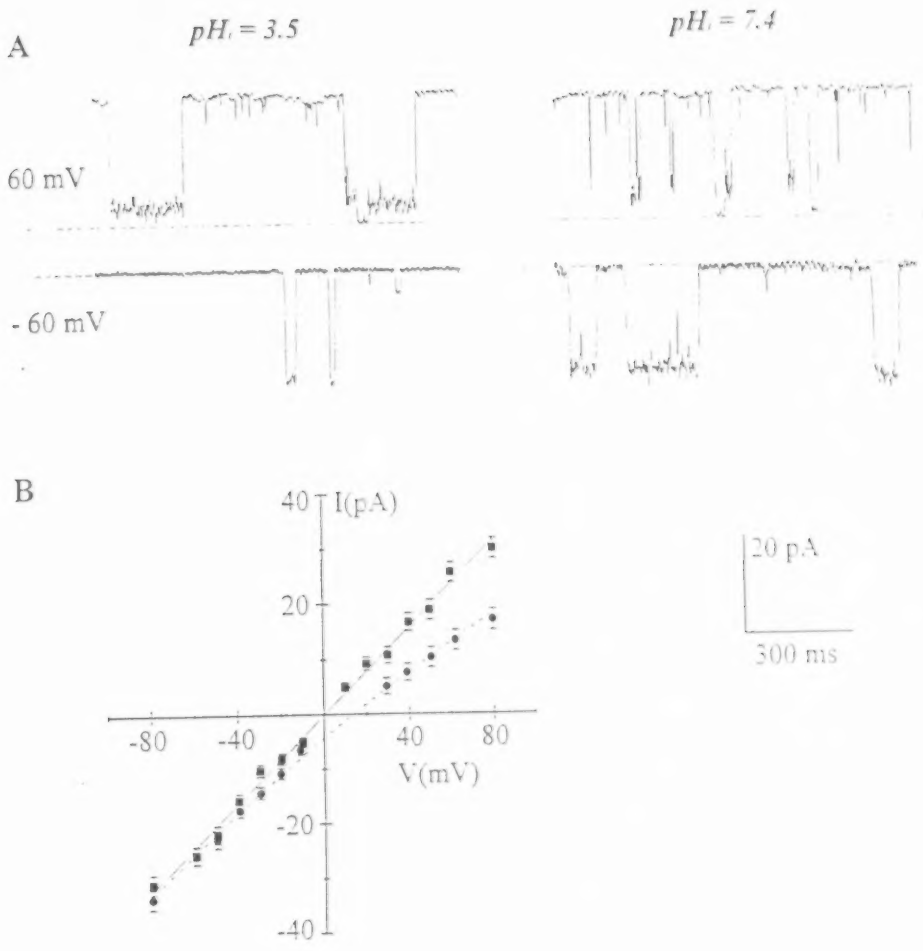

Fig. 5.1. A) Single-channel recordings of a large conductance Cl^- channel at ± 60 mV. Dotted lines indicate closed conditions in all cases. Bath pH is shown above the tracings. B) Current-voltage relationships under symmetrical pipette/bath 140 mM NaCl (squares) and asymmetrical 70/140 mM NaCl (circles) conditions. The data are presented as the means ±SD of 12 recordings in symmetrical and 4 in asymmetrical solutions (pH=7.4). The solid line shows a linear regression fit giving a channel conductance of 397.4 ±16.1 pS. The dashed line represents the best fit of GHK equation (1) under non-symmetrical NaCl conditions and gives a channel permeability ratio of P_{Na}/P_{Cl}=0.11 ± 0.04.

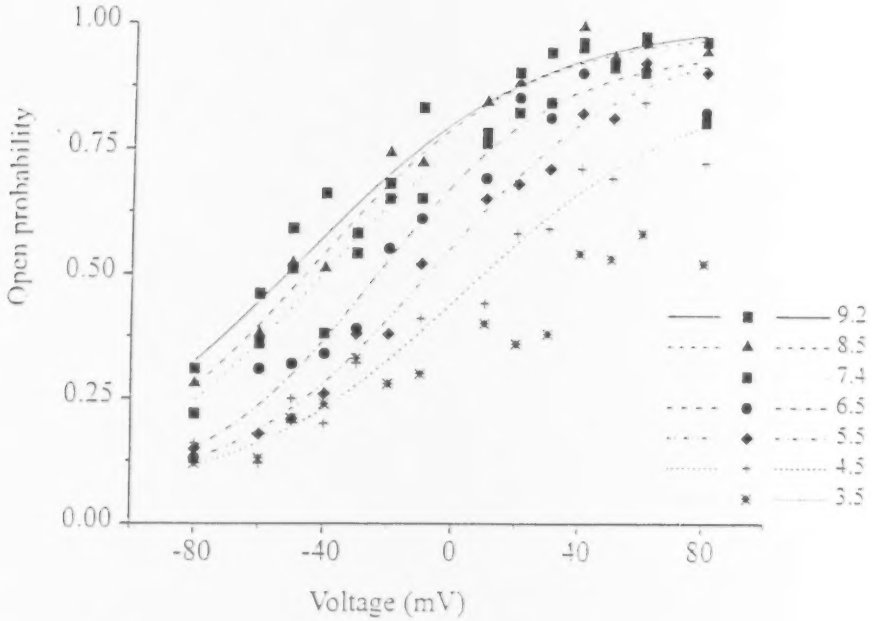

Fig. 2 A) Single channel open probability versus voltage at different bath pH values. The data represent mean values from 6(3.5), 6(4.5), 5(5.5), 8(6.5), 11(7.4), 3(8.5) and 8(9.2) different experiments. Standard deviations varied between 0.08 and 0.29, and were omitted for clarity. The lines through the data were obtained from equation (5)

CONCLUSION

Using the patch clamp technique and reconstitution of ion channel proteins into giant liposome, several groups of Cl^- channels from the apical membrane of epithelial cells were identified. The most commonly observed chloride channels were similar to those observed in native epithelial tissues. The permeability sequences and anomalous mole-fraction behaviors were examined. The linear 20 pS Cl^- channel had the halide permeability sequence $Cl^->I^-\geq Br^->F^-$, and showed anomalous mole-fraction behavior in solutions containing different proportions of Cl^- and F^- ions. The outwardly rectifying Cl^- channel had the halide permeability sequence $I^->Br^->Cl^->F^-$, and also showed anomalous mole-fraction behavior, indicating that both these channels probably contain multi-ion pores. The voltage-dependent anion channel showed at least five different substates, had a conductance of 390 pS in the main state, and it showed fast and slow opening and closing kinetics (<1 ms to few second). It did not show significant selectivity of the halides, and there was no deviation from a linear dependence of relative current on molar fractions, indicating relatively simple peameation through the pore. As these channels have been previously demonstrated in the native tissue, and the relative ease in obtaining good ion channel recordings, we suggests that the biochemical procedures for isolation of ion channels and the subsequent reconstitution of these channels into giant liposomes are useful tools for the study of ion channels.

Detailed studies were carried out in several Cl^- channels. The first study dealed with the regulation of a 100-120 pS channel by G-protein. Application of GTPγS or NaF^- to excised patches revealed the existence of a novel type of Cl^- channel from the apical

membrane of bovine tracheal epithelial cells. The channel was regulated by G-protein in a membrane-delimited manner, and had a linear-current-voltage relationship with conductance of 100-120 pS. The channel was inhibited by the stilbene disulphonates SITS and DNDS without changing the conductance. With SITS the channel opening become voltage-dependence and this indicated the possible involvement of lysine residues in the channel permeation pathway. The channel was insensitive to alkaline phosphatase and to the specific inhibitors of protein phophatases type I and 2A, okadaic acid and calyculin A, indicating that these protein phosphatases were not involved in the channel regulation. NaF was unable to activate Cl^- channels in the presence of the aluminum chelator, deferoxamine mesylate. This indicates that Al^{3+} ions play an important role in chloride channel activation by fluoride. The channels could be activated by GTPγS or by NaF in the presence of the phospholipase A_2 inhibitor quinacrine, indicating this enzyme is not involved in channel regulation.

The second study examined the effects of pH changes on the activity of a large (≈390 pS) Cl^- channel from bovine tracheal epithelial cells. Alkaline pH in the range 7 to 9.2 had no appreciable effect on channel conductance and gating, however, acidic pH significantly reduced channel open probability, and raised the voltage required to open the channel. Analysis of channel activity in the acidic pH range has suggested that at least one charged group on the channel with an apparent pK of 6.09, and that this might control the activity of this large epithelial Cl^- channel. Additionally, the lack of channel conductance changes in a wide pH range suggested that the internal protonation site was far removed from the channel permeation pathway.

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